Hematopoietic stem cell expansion

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HEMATOPOIETIC STEM CELL EXPANSION

Marta Anna Walasek

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For my dearest mother

Dla mamy

Paranimfen:	Bertien Dethmers-Ausema
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CHAPTER 1

INTRODUCTION & OUTLINE OF THE THESIS

INTRODUCTION

Hematopoiesis and hematopoietic stem cells. Hematopoiesis is the process of blood cell development and formation. Blood contains many specialized mature cell types, such as red blood cells (erythrocytes), platelets (thrombocytes), granulocytes, macrophages, and B- and T-lymphocytes, that all play important roles in the function of an organism, ranging from oxygen supply to all tissues, to defense against pathogens. Since these cells have a limited lifespan, they have to be continuously replenished to sustain steady state levels in the peripheral circulation. Approximately 10^{11}–10^{12} new blood cells are produced daily, by a small population of hematopoietic stem cells (HSCs).[1-3] This multipotent cell population resides on the top of the hematopoietic hierarchy and through a cascade of differentiation steps HSCs can replenish all mature blood lineages. As a result of early commitment and proliferation, HSC give rise to multipotent progenitor cells (MPP), which become further restricted in their developmental potential and differentiate into progenitors of lymphoid or myeloid or mixed lympho-myeloid lineage (CLP, CMP or LMPP, respectively).[4-7] Progenitors of myeloid lineage commit further into granulocyte/macrophage progenitors (GMP) that arise from CMP or LMPP, or into megakaryocytic/erythroid progenitors (MEP) that originate from CMP. Eventually these distinct committed progenitors differentiate into all mature blood cells types, completing the hematopoietic differentiation cascade (Figure 1). For lifelong hematopoiesis the maintenance of the HSC pool is crucial and relies on long-term self-renewal potential, a unique feature of stem cells that is gradually lost as cell commit into one of the blood lineages. Thus, the ability of multilineage differentiation and self-renewal are the hallmark features of HSCs. The intricate balance between these two stem cell fates is essential to maintain homeostasis between the number of stem cells and their differentiated progeny, and is required to enable rapid and robust response to physiological stresses, including blood loss, infection and injury. Properties of HSCs render these cells an attractive source for a wide range of stem cell-based therapies where restoration of blood cells or introduction of normal hematopoiesis is needed, such as in malignancies, bone marrow failure, immunodeficiencies and genetic disorders. However, progress in the clinical applications of HSCs has been restricted by the limited stem cell numbers available for transplantations from some stem cell sources, such as umbilical cord blood. Therefore attempts have been made to amplify HSC numbers *ex vivo*, prior their transplantations. The success of *ex vivo* stem cell expansion protocols is dependent on our ability to manipulate the behavior of HSCs; to stimulate cell proliferation with concomitant maintenance of self-renewal potential. Development of such protocols requires methods for identification and characterization of HSC, understanding of the mechanisms governing HSC self-renewal and differentiation decisions, and ultimately defining key factors stimulating HSC self-renewal divisions.

Characterization of hematopoietic stem cells. Murine HSCs were identified for the first time by Till and McCulloch more than 50 years ago[1,2] , and nowadays are the best characterized adult stem cells in terms of markers that allow their purification, but also in terms of assays for functional potential. Tools allowing HSC functional characterization, in combination with development of selection methods based on cell surface markers, advanced the prospective identification and isolation of stem cells. Changes in cell surface marker expression reflect HSC differentiation

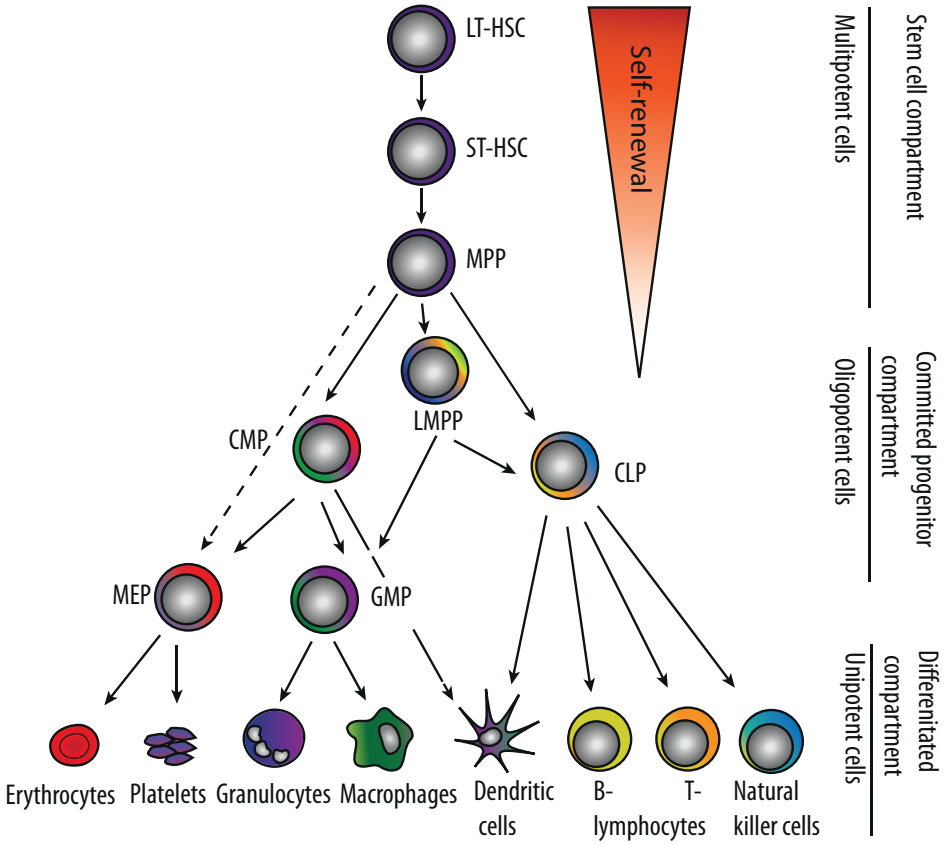

Figure 1. Model of hematopoiesis. On the top of hematopoietic differentiation hierarchy resides the multipotent stem cell compartment, consisting of long-term (LT-HSC) and short-term (ST-HSC) HSCs. Self-renewal capacity is restricted to this stem cell compartment and gradually decreases moving downwards the hierarchy (indicated by the triangle). Upon differentiation, HSCs give rise to all mature blood cells through a cascade of distinct and developmentally restricted progenitor stages, including multipotent progenitors (MPP), lymphoid primed multipotent progenitors (LMPP), common lymphoid progenitors (CLP), common myeloid progenitors (CMP), granulocyte/macrophage progenitors (GMP) and megakaryotic/erythroid progenitors (MEP).

and allow distinction of separate intermediate stages along the differentiation hierarchy. Studies have indicated that murine HSCs are contained within a cell population that lacks the expression of surface markers specific for mature blood cells (so-called lineage markers), but do express both Stem Cell Antigen-1 (Sca-1) and c-kit.[8,9] Therefore, the HSC-containing subset of bone marrow is known as the LSK population. This compartment can be further subdivided in primitive, long-term (LT) HSC (representing only ~2% of LSK population), short-term (ST) HSC and multipotent progenitors (MPPs) based on the expression profile of additional markers, such as CD34, Flk-2/Flt-3, SLAM (CD48, CD150), and EPCR (CD201) (Figure 1).[10-15] Alternatively, the HSC-enriched cell population can be identified based on the ability to efflux DNA-binding

vital dyes such as Hoechst 33342 and the mitochondrial activity marker Rhodamine 123, marking the so-called side population (SP).[16-18] However, the most important determinant in stem cell characterization is their functional potential. A number of assays have been developed to monitor hematopoietic stem and progenitor cell (HSPC) activity *in vitro* and *in vivo*. *In vitro* assays to measure HSPC potential include the colony forming unit assay (CFU), the cobblestone-area forming cell assay (CAFC), and the long-term culture-initiating cell assay (LTC-IC).[19,20] These assays allow for a relatively (compared to *in vivo* assays) quick estimate of the frequency and activity of stem and/or progenitor cells in a test cell population, and offer a method for screening the effects of a certain treatment (such as new compounds) on HSC function. Nonetheless, the ultimate proof of HSC function can only be obtained using the *in vivo* transplantation assay, measuring both self-renewal and multilineage differentiation capacity of test cells after transfer to a myeloablated host. Long-term reconstitution experiments following transplantation of a single purified cell provided functional proof of the existence of adult HSCs and showed their enormous proliferative and self-renewal potential.[10] Experimental breakthroughs, including high-purity HSC isolation and separation of distinct differentiation stages, together with well established functional assays, greatly facilitated the study of key mechanisms and molecules involved in regulation of HSC self-renewal and differentiation.

Hematopoietic stem cell fate decisions and stem cell expansion. Quiescence, apoptosis, self-renewal and differentiation are all possible fates of HSCs. Elucidation of factors that control HSCs fate, specifically the ones promoting self-renewal and maintaining stem cells in an undifferentiated state, has been a long-standing goal of stem cell biology, as these factors potentially represent a means for promoting expansion of HSCs *ex vivo*. Self-renewal can be defined as a cell division in which one (maintenance) or both (expansion) of the daughter cells remain undifferentiated and retain the same properties as a parent cell. It is believed that in order for a stem cell to self-renew it must integrate survival signals, proliferation control and it has to repress alternative transcriptional fates, such as commitment into a certain lineage. *In vivo,* HSCs are exposed to a complex interplay of diverse signals, both cell autonomous (intrinsic) but also cell non-autonomous (extrinsic), ultimately determining the fate of dividing HSC. Gene manipulation and genome-wide expression profiling studies have proven to be important tools in delineating the mechanism playing a role in the regulation of HSC fate decisions. Several distinct cell intrinsic factors, such as chromatin remodelers[21,22], transcription factors[23-26], microRNAs[27-31], signal transducers[32], and anti-apoptotic signals[33,34], but also signals from the rich milieu of the cell, molecules produced by HSC microenvironment (so-called niche), including growth factors[35-40] and signals involved in HSC ontogeny[41-48], have been shown to be able to affect HSC fates. However at present, a holistic perspective of how stem cells balance self-renewal versus differentiation decisions is lacking, and the regulatory networks orchestrating HSC self-renewal decisions remain to be determined. A remaining challenge is insight into if and how cell intrinsic and extrinsic signals are integrated with each other. Extrinsic control of HSC self-renewal and differentiation might be the preferred approach in clinical *ex vivo* HSC expansion protocols, since genetic manipulation of the cell might result in undesired clinical outcomes, for instance development of leukemia. However, in most cases extrinsic manipulation of self-renewal has proven to be difficult, since HSCs proliferation *in vitro*

inevitably leads to hematopoietic differentiation or death, with an overall loss of transplantable HSC activity. Therefore, many studies focus on the search for extrinsic molecules stimulating HSC self-renewal programs. More recently, the activity of non-hematopoietic factors, such as neuronal factors[49] or chemical compounds[50-53] was assessed, which opened new possibilities for *ex vivo* HSC expansion protocols. Nonetheless, the quest for (combinations of) factors that efficiently induce extensive HSC proliferation while simultaneously stimulating HSC self-renewal and/or inhibiting HSCs differentiation in culture is still continuing.

OUTLINE OF THE THESIS

In this thesis we aimed to explore the potential of distinct intrinsic and extrinsic factors to regulate HSC self-renewal and differentiation with the ultimate purpose of *ex vivo* stem cell expansion.

Chapter 2 provides an introduction to the *ex vivo* stem cell expansion concept. It contains an overview of the known factors controlling HSC self-renewal, with the emphasis on extrinsic control of HSPC fate. Previous expansion approaches and more recent directions in HSPC expansion attempts are reviewed in this chapter.

Chapter 3 describes a study aimed to identify new intrinsic regulators of hematopoiesis. In this chapter we profiled microRNA expression patterns during hematopoietic differentiation in two distinct mouse strains (C57BL/6 and DBA/2), and we identified microRNAs specific for HSPCs and correlating with HSPC frequency. Of special interest was an evolutionary conserved microRNA cluster 99b/let-7e/125a. To study whether members of this cluster could control HSPC fate decisions, the miRNAs were overexpressed in HSPCs, and we show that this led to initial expansion of primitive cells, but in the long-term resulted in stem cell exhaustion. Finally, using a bioinformatics approach, we identified candidate downstream targets through which miR-125a may be able to modulate HSPC fate.

Chapter 4 illustrates the potential of small molecule compounds to manipulate HSPC fate decisions in culture. In this study, the combined effects of valproic acid (VPA) and lithium (Li) on HSPC proliferation, differentiation and self-renewal under strong differentiation pressure were investigated. We showed that the combination of these two compounds displayed strong anti-differentiation effects at the level of uncommitted and committed progenitors, both at the biological and molecular level. The combination of VPA and Li synergistically preserved expression of stem cell-related genes and repressed genes involved in differentiation.

In **Chapter 5** we studied the molecular mechanism underlying the enhancing effects of VPA on HSPC by profiling early response VPA mRNA targets. We identified Sca-1 as a direct VPA targets and showed that Sca-1 can be readily, quickly and efficiently re-induced on distinct hematopoietic cells subsets by VPA. Finally, we demonstrated that re-activation of Sca-1 expression on hematopoietic cells correlated with enhanced self-renewal capacity. Although, Sca-1 was not necessary for the biologic effects of VPA, as shown when Sca-1 deficient cells were used, it marked HDI-responsive cells with enhanced functionality.

Chapter 6 summarizes the findings described in the previous chapters, and discusses a perspective for future research.

REFERENCES

1. McCulloch EA and Till JE. The Radiation Sensitivity of Normal Mouse Bone Marrow Cells, Determined by Quantitative Marrow Transplantation into Irradiated Mice. *Radiat.Res*. 1960;13:115-125.
2. Till J and McCulloch E. A Direct Measurement of the Radiation Sensitivity of Normal Mouse Bone Marrow Cells. *Radiat Res*. 1961;14:213-222.
3. Bryder D, Rossi DJ, Weissman IL. Hematopoietic Stem Cells: The Paradigmatic Tissue-Specific Stem Cell. *Am J Pathol*. 2006;169:338-346.
4. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*. 2000;404:193-197.
5. Kondo M, Weissman IL, Akashi K. Identification of Clonogenic Common Lymphoid Progenitors in Mouse Bone Marrow. *Cell*. 1997;91:661-672.
6. Adolfsson J, Månsson R, Buza-Vidas N, et al. Identification of Flt3+ Lympho-Myeloid Stem Cells Lacking Erythro-Megakaryocytic Potential: A Revised Road Map for Adult Blood Lineage Commitment. *Cell*. 2005;121:295-306.
7. Månsson R, Hultquist A, Luc S, et al. Molecular Evidence for Hierarchical Transcriptional Lineage Priming in Fetal and Adult Stem Cells and Multipotent Progenitors. *Immunity*. 2007;26:407-419.
8. Spangrude G, Heimfeld S, Weissman I. Purification and characterization of mouse hematopoietic stem cells. *Science*. 1988;241:58-62.
9. Okada S, Nakauchi H, Nagayoshi K, Nishikawa S, Miura Y, Suda T. In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. *Blood*. 1992;80:3044-3050.
10. Osawa M, Hanada K, Hamada H, Nakauchi H. Long-Term Lymphohematopoietic Reconstitution by a Single CD34-Low/Negative Hematopoietic Stem Cell. *Science*. 1996;273:242-245.
11. Christensen JL and Weissman IL. Flk-2 is a marker in hematopoietic stem cell differentiation: A simple method to isolate long-term stem cells. *Proc Natl Acad Sci*. 2001;98:14541-14546.
12. Adolfsson J, Borge OJ, Bryder D, et al. U\pregulation of Flt3 Expression within the Bone Marrow LinSca1+c-kit+ Stem Cell Compartment Is Accompanied by Loss of Self-Renewal Capacity. *Immunity*. 2001;15:659-669.
13. Kiel MJ, Yilmaz ÖH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ. SLAM Family Receptors Distinguish Hematopoietic Stem and Progenitor Cells and Reveal Endothelial Niches for Stem Cells. *Cell*. 2005;121:1109-1121.
14. Balazs AB, Fabian AJ, Esmon CT, Mulligan RC. Endothelial protein C receptor (CD201) explicitly identifies hematopoietic stem cells in murine bone marrow. *Blood*. 2006;107:2317-2321.
15. Dykstra B, Olthof S, Schreuder J, Ritsema M, de Haan G. Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells. *J Exp Med*. 2011;208:2691-2703.
16. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med*. 1996;183:1797-1806.
17. Li CL and Johnson GR. Rhodamine123 reveals heterogeneity within murine Lin-, Sca-1+ hemopoietic stem cells. *J Exp Med*. 1992;175:1443-1447.
18. Spangrude GJ and Johnson GR. Resting and activated subsets of mouse multipotent hematopoietic stem cells. *Proc Natl Acad Sci*. 1990;87:7433-7437.
19. van Os R, Kamminga LM, de Haan G. Stem Cell Assays: Something Old, Something New, Something Borrowed. *Stem Cells*. 2004;22:1181-1190.
20. van Os RP, Dethmers-Ausema B, and de Haan G. In Vitro Assays for Cobblestone Area-Forming Cells, LTC-IC, and CFU-C. *Methods Mol Biol*. 2008;430:143-157.
21. Kamminga LM, Bystrykh LV, de Boer A, et al. The Polycomb group gene Ezh2 prevents hematopoietic stem cell exhaustion. *Blood*. 2006;107:2170-2179.
22. Rizo A, Dontje B, Vellenga E, de Haan G, Schuringa JJ. Long-term maintenance of human hematopoietic stem/progenitor cells by expression of BMI1. *Blood*. 2008;111:2621-2630.
23. Antonchuk J, Sauvageau G, Humphries RK. HOXB4-Induced Expansion of Adult Hematopoietic Stem Cells Ex Vivo. *Cell*. 2002;109:39-45.
24. Fischbach NA, Rozenfeld S, Shen W, et al. HOXB6 overexpression in murine bone marrow immortalizes a myelomonocytic precursor in vitro and causes hematopoietic stem cell expansion and acute myeloid leukemia in vivo. *Blood*. 2005;105:1456-1466.
25. Zeng H, Yucel R, Kosan C, Klein-Hitpass L, Moroy T. Transcription factor Gfi1 regulates self-renewal and engraftment of hematopoietic stem cells. *EMBO J*. 2004;23:4116-4125.
26. Kato Y, Iwama A, Tadokoro Y, et al. Selective activation of STAT5 unveils its role in stem cell self-renewal in normal and leukemic hematopoiesis. *J Exp Med*. 2005;202:169-179.
27. Gerrits A, Walasek MA, Olthof S, et al. Genetic screen identifies microRNA cluster 99b/let-7e/125a as a regulator of primitive hematopoietic cells. *Blood*. 2011.
28. Guo S, Lu J, Schlanger R, et al. MicroRNA miR-125a controls hematopoietic stem cell number. *Proc Natl Acad Sci*. 2010;107:14229-14234.
29. Han Y, Park CY, Bhagat G, et al. microRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia. *J Exp Med*. 2010;207:475-489.
30. Ooi AGL, Sahoo D, Adorno M, Wang Y, Weissman IL, Park CY. MicroRNA-125b expands hematopoietic stem cells and enriches for the lymphoid-balanced and lymphoid-biased subsets. *Proc Natl Acad Sci*. 2010;107:21505-21510.
31. Bousquet M, Harris MH, Zhou B, Lodish HF. MicroRNA miR-125b causes leukemia. *Proc Natl Acad Sci*. 2010;107:21558-21563.
32. Zhang J, Grindley JC, Yin T, et al. PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. *Nature*. 2006;441:518-522.

33. Domen J, Cheshier SH, Weissman IL. The Role of Apoptosis in the Regulation of Hematopoietic Stem Cells: overexpression of BCL-2 increases both their number and repopulation potential. *J Exp Med*. 2000;191:253-264.
34. Opferman JT, Iwasaki H, Ong CC, et al. Obligate Role of Anti-Apoptotic MCL-1 in the Survival of Hematopoietic Stem Cells. *Science*. 2005;307:1101-1104.
35. Zhang CC and Lodish HF. Insulin-like growth factor 2 expressed in a novel fetal liver cell population is a growth factor for hematopoietic stem cells. Blood. 2004;103:2513-2521.
36. Zhang CC, Kaba M, Ge G, et al. Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. *Nat Med*. 2006;12:240-245.
37. Yeoh JSG, van Os R, Weersing E, et al. Fibroblast Growth Factor-1 and -2 Preserve Long-Term Repopulating Ability of Hematopoietic Stem Cells in Serum-Free Cultures. *Stem Cells*. 2006;24:1564-1572.
38. Qian H, Buza-Vidas N, Hyland CD, et al. Critical Role of Thrombopoietin in Maintaining Adult Quiescent Hematopoietic Stem Cells. *Cell Stem Cell*. 2007;1:671-684.
39. Miller CL and Eaves CJ. Expansion in vitro of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability. *Proc Natl Acad Sci*. 1997;94:13648-13653.
40. Audet J, Miller CL, Eaves CJ, Piret JM. Common and distinct features of cytokine effects on hematopoietic stem and progenitor cells revealed by dose-response surface analysis. *Biotechnol Bioeng*. 2002;80:393-404.
41. Bhardwaj G, Murdoch B, Wu D, et al. Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat Immunol*. 2001;2:172-180.
42. Bhatia M, Bonnet D, Wu D, et al. Bone Morphogenetic Proteins Regulate the Developmental Program of Human Hematopoietic Stem Cells. *J Exp Med*. 1999;189:1139-1148.
43. Duncan AW, Rattis FM, DiMascio LN, et al. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nat Immunol*. 2005;6:314-322.
44. Luis T, Naber BE, Roozen PC, et al. Canonical Wnt Signaling Regulates Hematopoiesis in a Dosage-Dependent Fashion. *Cell Stem Cell*. 2011;9:345-356.
45. Murdoch B, Chadwick K, Martin M, et al. Wnt-5A augments repopulating capacity and primitive hematopoietic development of human blood stem cells in vivo. *Proc Natl Acad Sci*. 2003;100:3422-3427.
46. Perry JM, He XC, Sugimura R, et al. Cooperation between both Wnt/β-catenin and PTEN/PI3K/Akt signaling promotes primitive hematopoietic stem cell self-renewal and expansion. *Genes Dev*. 2011;25:1928-1942.
47. Reya T, Duncan AW, Ailles L, et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature*. 2003;423:409-414.
48. Varnum-Finney B, Purton LE, Yu M, et al. The Notch Ligand, Jagged-1, Influences the Development of Primitive Hematopoietic Precursor Cells. *Blood*. 1998;91:4084-4091.
49. Himburg HA, Muramoto GG, Daher P, et al. Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells. *Nat Med*. 2010;16:475-482.
50. Araki H, Yoshinaga K, Boccuni P, Zhao Y, Hoffman R, Mahmud N. Chromatin-modifying agents permit human hematopoietic stem cells to undergo multiple cell divisions while retaining their repopulating potential. *Blood*. 2007;109:3570-3578.
51. Boitano AE, Wang J, Romeo R, et al. Aryl Hydrocarbon Receptor Antagonists Promote the Expansion of Human Hematopoietic Stem Cells. *Science*. 2010;329:1345-1348.
52. Bug G, Gül H, Schwarz K, et al. Valproic Acid Stimulates Proliferation and Self-renewal of Hematopoietic Stem Cells. *Cancer Res*. 2005;65:2537-2541.
53. Li W and Ding S. Small molecules that modulate embryonic stem cell fate and somatic cell reprogramming. *Trends Pharmacol Sci*. 2010;31:36-45.

CHAPTER 2

HEMATOPOIETIC STEM CELL EXPANSION: CHALLENGES AND OPPORTUNITIES

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ABSTRACT

Attempts to improve hematopoietic reconstitution and engraftment potential of *ex vivo*-expanded hematopoietic stem and progenitor cells (HSPCs) have been largely unsuccessful due to the inability to generate sufficient stem cell numbers and to excessive differentiation of the starting cell population. Although hematopoietic stem cells (HSCs) will rapidly expand after *in vivo* transplantation, experience from *in vitro* studies indicates that control of HSPC self-renewal and differentiation in culture remains difficult. Protocols that are based on hematopoietic cytokines have failed to support reliable amplification of immature stem cells in culture, suggesting that additional factors are required. In recent years, several novel factors, including developmental factors and chemical compounds, have been reported to affect HSC self-renewal and improve *ex vivo* stem cell expansion protocols. Here, we highlight early expansion attempts and review recent development in the extrinsic control of HSPC fate *in vitro*.

INTRODUCTION

The two defining features of hematopoietic stem cells (HSCs), self-renewal and multilineage differentiation, render these cells an attractive source for stem cell-based therapies. HSC transplantation can be a life-saving procedure in the treatment of a broad spectrum of disorders, including hematologic, immune, and genetic diseases. Bone marrow (BM) and mobilized peripheral blood stem cells are the most common HSC sources for transplantation; however, their use is restricted by the low availability of suitable human leukocyte antigen (HLA)-matched donors. An alternative approach, allowing the use of more rapidly available partly mismatched donors, involves the use of umbilical cord blood (UCB), which has been shown to be a rich source of HSCs. However, the stem cell number in a single UCB unit is not sufficient for transplantation into an adult.[1] These two issues, the lack of HLA-matched donors and the low number of stem cells available from common HSC sources for transplantation, especially in the case of UCB, present serious limitations to HSC transplantations. Clinical experience has shown that the stem cell dose (measured as $CD34^+$ cell number) is related to patient survival and time required for engraftment,[1-3] indicating that amplification of hematopoietic stem and progenitor cells (HSPCs) is expected to be highly beneficial for their clinical applications.

HSCs are a rare population of cells, representing less than 0.01% of all cells in the BM. During homeostasis, the stem cell pool is maintained at a relatively constant level. In contrast, several studies have shown that during hematopoietic stress, such as serial transplantations, HSCs can and will self-renew extensively. Studies by Pawliuk *et al.*, Iscove, and Nawa have shown that following injection into lethally irradiated recipients, HSC numbers increased by 10- to 20-fold.[4,5] When these cells were serially transplanted, the initial number of transplanted HSCs increased cumulatively by 8,400-fold after four successive passages.[5] Furthermore, it has been shown that a single purified HSC can reconstitute long-term hematopoiesis of lethally irradiated recipients, indicating robust *in vivo* self-replication capacity.[6] These studies have unequivocally demonstrated that HSCs can massively expand, and suggest that *in vivo* stem cells are exposed to specific factors/signals that promote their self-renewal and amplification.

Although HSC self-renewal divisions *in vivo* clearly occur, induction of self-renewal *in vitro* has been difficult. Even after several decades of research, the quest for factors that stimulate self-renewal *in vitro* is still continuing. The aim of these studies is to define the culture conditions that will support unlimited *ex vivo* expansion of HSCs from any source. To achieve this goal, stimulation of symmetrical self-renewal divisions over differentiation divisions is required (Figure 1). Although many efforts have been made to expand stem cells, extensive *in vitro* amplification of HSCs without loss of their repopulating potential has not yet been achieved. Nonetheless, several cell-intrinsic and cell-extrinsic factors have been identified and shown to have potential to expand HSCs in culture. Here, we summarize how *ex vivo* HSC expansion protocols have evolved, briefly reviewing the early expansion attempts and then focusing on recent expansion approaches involving small molecules.

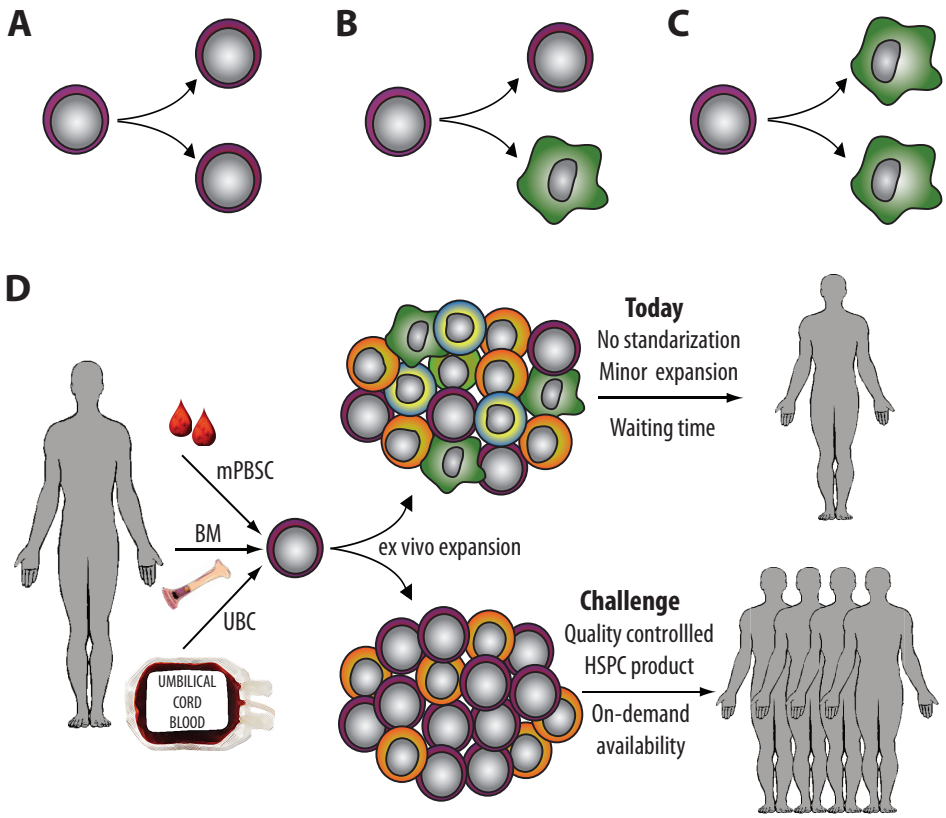

Figure 1. HSC fate outcomes define the composition of *ex vivo*-expanded product. (A) Symmetrical HSC self-renewal division. As a result of this division the parent stem cell give rise to two daughter cells with the identical properties as the parent cell, increasing the stem cell pool. Occurring during development of hematopoietic system in fetal liver and upon BM transplantation, remains difficult to sustain during *ex vivo* stem cell culture. (B) Asymmetrical HSC self-renewal division. Following asymmetrical self-renewal the parent stem cell generates one identical daughter cells and a progeny committed to differentiation, thus preserving stem cell numbers. Occurring during hematopoietic homeostasis in adulthood and during *ex vivo* HSC expansion culture supporting stem cell maintenance. (C) Symmetrical HSC division. Outcome of symmetrical cell division is generation of two committed to differentiation daughter cells, leading to stem cell depletion. Occurring in a pathological conditions characterized by stem cell exhaustion and usually during *ex vivo* HSC culture. (D) HSC expansion today and the 'Holy Grail' of expansion research. Ex *vivo* HSC expansion protocols available today cannot efficiently support symmetrical self-renewal divisions of HSC resulting (optimistically) in maintenance or minor amplification of stem cells and expansion of differentiated progeny. It remains unclear whether stem cells from any source benefit from up-to-date expansion protocols, resulting in lack of standardized on-demand expanded product. The challenge remains to expand undifferentiated HSC (via stimulation of symmetrical self-renewal divisions) in numbers sufficient for therapy of adult patients. This would allow development of clinically relevant and quality-controlled HSPC expanded product that can be supplied upon demand.

REGULATION OF HSC SELF-RENEWAL BY INTRINSIC FACTORS

One of the remaining fundamental questions of stem cell biology is how self-renewal is regulated. Answers to this question would also substantially contribute to the success of *ex vivo* HSC expansion protocols. Valuable knowledge about factors influencing self-renewal has been gained from gene manipulation studies, which have identified multiple proteins that play an important role in the regulation of HSC self-renewal, including transcription factors, epigenetic modifiers, and cell cycle regulators (Figure 2) (reviewed in Refs. 7 and 8). Here, we will only highlight a few examples, representing different classes of proteins, such as DNA-binding, chromatin-binding, and RNA-binding factors.

One of the first genes described to play a role in HSC fate determination was *HoxB4*. Ectopic expression of this transcription factor resulted in robust (40-fold) expansion of transplantable murine HSCs, but did not coincide with the development of leukemia.[9,10] HSC amplification could also be stimulated by an extrinsically delivered TAT-HoxB4 fusion protein, although the HSC expansion levels achieved with this fusion protein were much lower compared with ectopic protein expression.[11] Additionally, other members of HoxA and HoxB clusters (e.g., *HoxA4*, *HoxA9*, *HoxB6*) have also been shown to regulate fate determination of adult HSCs.[12-15] Overexpression of chromatin remodelers, such as members of the Polycomb group family of proteins (PcG), especially *Ezh2* or *Bmi1*, have been shown to modulate HSC activity by preventing stem cell exhaustion or augmenting self-renewal, respectively.[16-18] The recent discovery of noncoding RNAs, including microRNAs, added an additional level of regulation to the network controlling HSC fate determination.[19,20] MicroRNAs are short RNA molecules, 19-25 nucleotides in length that negatively regulate gene expression. Sequence-specific pairing of microRNA with targeted messenger RNA results in inhibition of its translation and/or triggers messenger RNA degradation.[19] MicroRNAs are predicted to modulate the expression of hundreds of messenger RNAs, profoundly affecting cellular gene expression profiles.[20;21] Thus, microRNAs might act as master regulators of cellular transcriptional programs. A growing body of studies indicates that microRNAs are critical players orchestrating developmental or differentiation stages of many distinct tissues, including hematopoiesis.[19;22;23] Ablation of Dicer, the enzyme required for microRNA processing resulted in an impairment of the HSC compartment in mice, indicating an essential role of microRNAs in HSCs maintenance.[24,25] Several microRNAs enriched in the stem cell compartment compared to bone-marrow cells, such as the miR-125 family and miR-29a, have been reported to affect HSPC self-renewal and differentiation decisions.[26-28] However, ectopic expressions of these microRNAs can lead to the development of leukemia or myelodysplastic syndrome.[24;26-30] Genetic studies aimed to decipher the mechanism of self-renewal have indicated that self-renewal is regulated at various levels and likely by a complex network of interacting stimuli.

Although the highest expansion levels have been reported after ectopic expression of cell-intrinsic genes, introduction of genetic material is undesired in clinical protocols since continuous activation of self-renewal may lead to potential malignant transformation or to stem cell exhaustion. Therefore, mild and/or transient activation of self-renewal by extrinsic factors or molecules that perturb the activity of intrinsic HSC self-renewal regulators (described above) might be a preferred tool for *ex vivo* stem cell expansion.

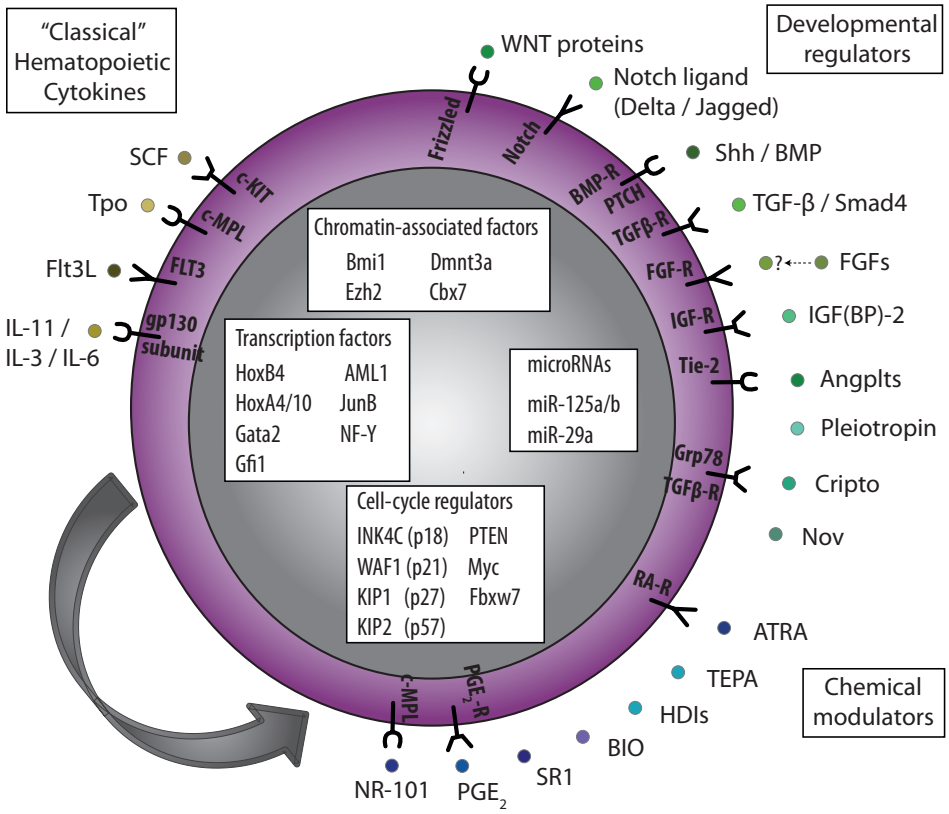

Figure 2. Cell-intrinsic and cell-extrinsic factors involved in HSC self-renewal. Self-renewal can be driven intrinsically by gene expression and can be regulated by extrinsic factors from environment. Cell-intrinsic regulation of HSC fate includes interplay between specific transcription factors, RNA/DNA-binding proteins and chromatin-associated factors. That network can be modulated by cell-extrinsic cues, like cytokines, developmental/growth factors and chemical compounds. These distinct stimuli create complex matrix of interactions that define the result of HSC fate suggesting that combination of distinct stimuli could be required for effective stimulation of self-renewal divisions and stem cell expansion.

EXTRINSIC REGULATORS OF HSC FATES

The first attempts to *ex vivo* amplify HSCs with extrinsic factors focused on the use of hematopoietic cytokines, many of which are produced by the *in vivo* HSC microenvironment. Although the role of cytokines in blood lineage development has been indisputably established, their regulatory role on HSC self-renewal has been questioned.[31-33] Multiple cytokines, including SCF, Tpo, Flt-3L, IL-11, IL-3, IL-6 and GM-CSF, and combinations of these, have been studied in *in vitro* HSC expansion protocols of mouse and human cells. For details of the effects of distinct cytokines on HSC characteristics in culture, we refer to the excellent review by Sauvageau *et al.*[33] Knowledge gained from cytokine studies indicates that whereas *in vitro* cell survival and proliferation can be efficiently stimulated by several cytokines (especially SCF and Tpo), these

cytokines by themselves are usually not sufficient to support self-renewal and they typically induce HSC differentiation, resulting at best in maintenance or modest stem cell amplification, but usually leading to progressive depletion of long-term repopulating cells.[34,35] Single cell cytokine cultures demonstrated that *in vivo* repopulating ability of mouse and human HSCs is gradually but inevitably lost, starting from the first *in vitro* cell division.[36-38] Nonetheless, the cocktails of SCF and Flt3L with IL-11 (mouse) or Tpo (mouse and human) was defined as the cytokine combination supporting the best HSPC survival, proliferation, and maintenance during *in vitro* culture, and is often used as the core cytokine mix to which other cytokines or expansion factors can be added (Table 1).[39-41] To conclude, efforts to improve the rate of HSPC engraftment by *ex vivo* cytokine-based expansion protocols has been largely unsuccessful, indicating the need of additional factors/molecules in order to support HSC self-renewal and amplification *in vitro*.

DEVELOPMENTAL REGULATORS AND HSC EXPANSION

Recently, factors that do not necessarily qualify as typical hematopoietic growth factors but rather play an instructive role during ontogeny (i.e., at a time when the hematopoietic system is first developed and is rapidly expanding) have been shown to also regulate the adult stem cell compartment. These factors stimulate several developmentally conserved pathways, such as wingless-type (Wnt), Notch, Sonic hedgehog (Shh), and fibroblast growth factor (FGF) signaling. Adult HSCs have been shown to express receptors that can activate all of the aforementioned pathways, indicating that these pathways may be employed to extrinsically control HSC fate in culture.

Wnt proteins. The Wnt signaling pathway, known by its role in embryogenesis and cancer, regulates cell-to-cell interactions in a wide variety of tissues. A role for Wnt has been convincingly demonstrated in homeostasis of the intestinal stem cell compartment,[42] and a role for Wnt has been explored in HSC fate regulation.[43] Wnts are secreted glycoproteins, expressed by the fetal and adult HSC microenvironment. Addition of soluble Wnt proteins, such as Wnt3a or Wnt5a, to liquid cultures or co-cultures with Wnt-transduced stroma, enhanced mouse and human HSC survival, proliferation, and self-renewal, as measured by engraftment rate after transplantation into lethally irradiated recipients.[44-47] Interestingly, it has recently been demonstrated that the level of Wnt signaling is critically important in regulating HSC self-renewal.[48] Binding of Wnt ligands to its receptor Frizzled triggers activation of the canonical signal transducer β-catenin and its translocation to the nucleus, leading to activation of Wnt target gene transcription.[49] Ectopic expression of a constitutively active form of β-catenin resulted in expansion of the stem cell pool.[43] In this study, upon activation of Wnt signaling, upregulation of *HoxB4* and *Notch1* were reported, suggesting a link between Wnt and Notch signaling pathways. Work of Duncan *et al*. supports cooperation of these two pathways in maintaining the HSC pool, thus demonstrating the importance of the Wnt pathway in cell proliferation and survival and a requirement of Notch for maintaining HSCs in an undifferentiated state.[50]

Notch pathway. Notch and Notch-ligands, such as Jagged-1, Jagged-2, and Delta-1, are expressed by HSCs and their microenvironment, indicating a potential role for Notch

Table 1. Summary of recent expansion protocols discussed in the text.

Molecule	S	Input cells	Supplements	Time	Effect			Mechanism	Ref.
					Assay	Fold over Control	Fold over Input		
FGFs	M	BM		3w	CAFC d-35 HSC frequency		↑ ↑		De Haan et al 2003 Yeoh et al 2006
IGF-2	M	BM SP CD45$^+$Sca1$^+$	SCF Tpo FGF-1	10d	CRU (HSC frequency)		↑ 24 (WBM) ↑ 7.8 (SP)		ZhangLodish 2004
Angplt	M	SP CD45$^+$Sca1$^+$	SCF Tpo FGF-1 IGF-2	10d	CRU (HSC frequency)		↑ 24 (Angptl2) ↑ 30 (Angptl3)	Angplts expansion effects are dependent on mammalin cell-specific post-translational modifications.	Zhang et al. 2006
Pleiotrophin	M H	LSKCD34- Lin-34$^+$38$^-$ UCB	SCF Flt3L Tpo	7d	LSK numbers CRU frequency (12 weeks p-tpx) % engraftment secondary tpx CFU % engraftment (4 weeks)	↑ 17 ↑ 6 ↑ 10 ↑ 4 ↑ 3	 ↑ 4 ↑ 10 ↑ 3		Himburg et al. 2010
ATRA	M	LSK	serum SCF Flt3L IL-6 IL-11	7d	% Lin-/Sca1$^+$ cells CFU-S (d8) Pre-CFU-S % engraftment Donor reconstitution (per 10^5 cells)	↑ 1.7/4 ↑ 12 ↓ 0.2 ↔ ↑ 5	↓ 0.5 ↑ 60 ↓ 0.4 ↓		Purton et al. 1999 Purton et al. 2000
TEPA	H	CD34$^+$ CD133$^+$ UCB	serum SCF Tpo Flt3L IL-6	3w 8w	total CD34$^+$CD38- cells total CD34$^+$ cells % engraftment	↑ 15 ↑ 10 ↑	 ↑		Pelet et al. 2004 Pelet et al. 2005 Pelet et al. 2004

VPA	H	$CD34^+$ UCB MPB BM	serum SCF Flt3L Tpo IL-3	1 - 3w	% $oCD34^+CD90^+$ cells % $CD34^+CD38^-$ cells CFU activity (plating efficiency) Replating CFU activity	↓ 1.5-6 ↑ 20-140 ↑ 85 ↓ 0.3-0.08 ↑ 5	↑ 16 ↑ 7	Slower cell cycle	de Felice et al. 2005
	H M	$CD34^+$ UCB BM LSK	SCF Flt3L Tpo IL-3	2-10d	total $CD34^+$ cell numbers CFU plating and replating activity CFU-S (d12) % engraftment	↑ ↑ ↑ ↑ 2.2		↓ p21 ↑ HoxB4 Activation of GSK3B-dependent signaling pathway	Bug et al. 2005
	H	UCB HSC	SCF Flt-3L Tpo	14d	total $CD45^+CD34^+$ cell numbers % $CD45^+CD34^+$ cells in S-phase SRC frequency	↑ 2.0 ↑ ↑ 6.0		↑ HoxB4	Seet et al. 2008
Chlamydocin	H	$CD34^+$ MPB	SCF Flt3L Tpo	24h	% engraftment (SRC)	↑ 4.0	↑ 2.0	↑ H4-Ac	Young et al 2004
5aza +TSA	H	$CD34^+$ UCB	serum SCF Flt3L MGDF IL-3	9d	Total $CD34^+$ cell numbers Total $CD34^+CD90^+$ cell numbers CFU activity CAFC activity SRC frequency of $CD34^+CD90^+$ cells	↑ 2.5 ↑ 6 ↑ 8 ↑ 7 ↑ 40	↑ 5 ↑ 12.5 ↑ 9.8 ↑ 11.5 ↑ 9	↑ H4-Ac	Araki et al. 2007
BIO	H	$CD34^+$ UBC	serum SCF Flt3L Tpo	5d	% cells in G_0/G_1 phase + Ki67dim CFU activity CFU replating activity Engraftment of expanded cells	↑ 28 + 1.5 ↑ 2 ↑ 6 ↑ 2	↑ 30 ↑ 15 ↔	↑ p57, Jagged-1, BMP8-b, RARRES2, Noggin ↑ nuclear ß-catenin ↓ cyclin D1, CEBPδ	Ko et al. 2011
	H	$CD34^+$ UBC	serum SCF Flt-3L Tpo	5d	Total $CD34^+$ cell number CFU % engraftment SRC frequency (6 weeks)	↓ ↓ ↔ ↑ 2.5		↑ ß-catenin nuclear-location ↑ c-myc, HoxB4 ↑ CXCR4 in stroma-adherent population	Holmes et al. 2008

continued on the next page

Molecule	S	Input cells	Supplements	Time	Effect			Mechanism	Ref.
					Assay	Fold over Control	Fold over Input		
SR1	H	$CD34^+$ MPB UBC	SCF Flt3L Tpo IL-6	3- 5w	Total $CD34^+$ cell numbers CFU Engraftment 1 week p-tpx SRC frequency Secondary SRC frequency	↑ 47 ↑ 65 ↑ 24 ↑ 14 ↑ 4	↑ 9500 ↑ 6 ↑ 17 ↑ 12	Antagonizing the aryl hydrocarbon receptor ↓ AHRR and CYP1B1	Boitano et al. 2010
PGE2	M H	WBM LSK		2h	CFU-S (d12) HSC frequency (CRU)	↑ 3 ↑ 2-4	↑	↑ Survivin, CXCR4 ↓caspase-3	North et al 2007 Hoggatt et al 2009
NR-101	H	$CD34^+$ $CD34^+38^-$ UCB		7d	Total $CD34^+CD38^-$ cell number % $CD34^+CD38^-$ cells in G1/G0-phase SRC frequency	↑ 2.3 ↑ 2.1 ↑ 2.3	↑ 2.9	Activation of STAT5, but not of STAT3. Accumulation of HIF-1α and enhanced activation of its targets.	Nishino et al. 2009

Abbreviations: S- species; M- mouse; H- human; FGFs, fibroblast growth factors; IGF-2, insulin growth factors 2; Angplts, angiopoietin-like proteins; ATRA, all-trans retonic acid; TEPA, tetra-ethylenepentamine; VPA, valproic acid; TSA, trichostatin A; 5aza, 5-aza-2'-deoxycytidine; BIO, 6-bromoindirubin-3'-oxime; SR1, StemRegenin1; PGE_2, prostaglandin E_2; BM, bone marrow; SP, side population; UCB, umbilical cord blood; MPB, mobilized peripheral blood; SCF, stem cell factor; Tpo, thromopoietin; Flt3L, Ftl3 ligand; IL, interleukin; MGDF, megakaryocyte growth and development factor; w, week; d, day; CAFC, cobblestone area forming cell; CFU(-S), colony forming cell (spleen); CRU, competitive repopulating unit; SRC, NOD/SCID-repopulating cell; H4-AC, histone 4 acetylation

interactions in hematopoiesis.[51] Studies on the involvement of the Notch pathway in HSC fate regulation demonstrated that an immobilized form of Delta-1, but not its soluble form, can expand murine and human HSPCs in culture. Incubation of human UCB $CD34^+$ cells with Delta-1 and cytokines resulted in increased numbers of NOD/SCID repopulating cells with secondary transplantation ability. Similar to the dependence of HSCs on Wnt-protein levels, the Notch-mediated effects were suggested to be dose-dependent, since HSPC expansion was observed only with lower Delta-1 concentrations.[52,53] A subsequent clinical phase I trial showed rapid neutrophil recovery, enhanced myeloid engraftment, and no signs of graft-versus-host disease (GVHD) following transplantation with Delta-1-expanded human UCB cells,[54] although it remains unclear whether the UCB cells benefitted from the expansion protocol.

Shh / BMP signals. Cytokine-based expansion cultures of HSCs can also be improved by addition of other developmental factors, namely by soluble Sonic hedgehog proteins (Shh). Following Shh stimulation of human $CD34^+CD38^-Lin^-$ cells, enhanced cell proliferation and increased recovery of NOD/SCID repopulating cells were reported.[55] Additionally, Shh-induced HSPC expansion was suggested to be dependent on downstream bone morphogenic protein-4 (BMP-4) signaling, because inhibition of BMP-4 abrogated Shh-induced expansion.[55] Since human HSCs express BMP receptors, addition of soluble BMP-4 could also improve human HSPC proliferation and maintenance following *in vitro* culture.[56]

FGF signaling. The observation that murine HSCs express high levels of FGF receptors has led to investigation of the role of FGF signaling in HSPC fate regulation. Among this large protein family, FGF-1 and FGF-2 in particular were shown to maintain/expand multilineage, serially-transplantable, long-term repopulating HSCs when added to cytokine- and serum-free cultures of unfractionated mouse BM.[57,58] However, addition of classical cytokines to these cultures and stimulation of proliferation overruled FGF-dependent stem cell preservation. Moreover, FGFs could not support the maintenance of an undifferentiated state of purified murine HSC in culture, and only appeared active when stromal elements were present, suggesting an indirect nature of the FGF effects.[58]

IGF and Angptls. Recently, several soluble proteins produced by the endothelium and fetal liver, including insulin-like growth factor 2 (IGF-2), IGF binding protein 2 (IGFBP-2), and angiopoietin-like proteins (Angptls), have been identified as growth factors that can enhance *ex vivo* expansion of HSCs. Zhang *et al*. reported that addition of IGF-2, a protein expressed in fetal liver cells, to serum-free cultures of fetal liver HSCs resulted in increased stem cell frequency in a transplantation setting.[59] Shortly afterward, it was reported that IGFBP-2 displayed similar or even better stem cell-supporting effects and that IGF-2 could be replaced by IGFBP-2 in *ex vivo* HSC expansion cultures.[60] These cultures also contained a combination of a developmental factor, FGF-1 (mentioned above), and two cytokines, SCF and Tpo. The efficiency of this expansion cocktail could be further enhanced by addition of angiopoietin-like proteins,[61] which include several members, such as Angplt2, Angplt3, Angplt5, and Angplt7. Culture of murine HSCs with these factors resulted in a 30-fold net expansion of long-term repopulating HSCs, one of the highest HSC amplifications achieved to date by extrinsic factors. Similar expansion levels have also been reported in human HSC cultures supplemented with the Lodish and Zhang cocktail.[60-62]

Pleiotrophin. Another novel microenvironmental factor, pleiotrophin, expressed by human brain endothelial cells, has recently been reported to be essential for HSC maintenance. Mouse HSCs cultured in the presence of Pleiotrophin displayed a net increase in *in vivo*-measured stem cell frequency and enhanced secondary repopulation activity upon serial transplantation. Pleiotrophin was also shown to improve *ex vivo* expansion of human $CD34^{+}CD38^{-}Lin^{-}$ HSCs, albeit to a lesser extent compared with murine cells. Pleiotrophin-mediated expansion was abrogated by addition of specific PI3K or Notch inhibitors to the culture medium, suggesting involvement of these pathways in the pleiotrophin stem cell-enhancing effects.[63] Additionally, whereas pleiotrophin deficiency did not show any *in vivo* steady-state hematopoietic effects, repression of pleiotrophin in stromal cells resulted in defective hematopoiesis.[64]

The studies discussed above indicate the potential usefulness of exploiting the molecular pathways involved in stem cell self-renewal in improving *ex vivo* HSPC amplification cultures. The challenge remains to integrate the various cell-intrinsic and cell-extrinsic signals into the regulatory networks controlling HSC fate outcomes and to decipher how these factors are linked with each other. Likely, the balance of various co-regulated stimuli, acting simultaneously or sequentially, is crucial for HSC fate outcomes, and the ability to mimic the orchestra of these signals may lie in the success of *in vitro* stem cell-expansion protocols.

SMALL MOLECULES

In recent years, evidence has accumulated suggesting that in addition to natural cytokines/factors chemical compounds can also have potent effects on stem cell expansion protocols (Table 1). Studies were launched to assess the ability of such compounds to control stem cell fates in culture, where the effects ranged from enhancement of stem cell expansion to stimulation of lineage-specific differentiation, including effects on iPS reprogramming efficiency.[65-67]

ATRA. Retinoic acid (RA), a derivative of vitamin A (retinol) normally present at low physiological concentration in serum, represents a naturally occurring small molecule.[68] Retinoids have been shown to play important roles in development, differentiation and homeostasis of a wide variety of cell types. The retinoic acid receptor agonist all-trans retinoic acid (ATRA) has a well-documented effect on hematopoietic cells in cancer treatment, where it can induce differentiation of leukemic cells.[69] Studies with RA receptor (RAR) deletion have revealed the importance of retinoids in normal hematopoiesis, since RAR-γ knock-out mice showed markedly reduced HSC numbers.[70] However, the utility of retinoids for *ex vivo* HSPC expansion is unclear. Purton *et al*. suggested that addition of ATRA to cultures of mouse HSPCs enhanced the maintenance of short- and long-term repopulating cells and augmented self-renewal in serial transplantation experiments.[71,72] Additionally, increased *Notch1* and *HoxB4* mRNA levels were observed upon ATRA stimulation, suggesting that ATRA effects could be at least partially mediated via known cell-intrinsic regulators of HSC self-renewal.[70] On the other hand, retinoic acid was also reported to negatively affect HSPC *ex vivo* expansion, since inhibition of retinoid signaling resulted in enhanced HSPC self-renewal *in vitro*.[73] Therefore, the effects of retinoids on *in vitro* HSPC self-renewal and differentiation need further investigation.

Copper chelator, TEPA. It has been shown that increased cellular copper levels can improve ATRA-stimulated differentiation of leukemic cells.[74] Additionally, ions such as magnesium, calcium, or copper (Cu) are known to play an important role in cell function, since disturbed homeostasis of these ions is associated with clinical symptoms. Copper malnutrition resulted in hematopoietic cell maturation arrest but did not affect progenitor cell development, which suggests that cellular Cu balance may play a role in HSPC proliferation and differentiation. Studies on modulation of cellular Cu content during *ex vivo* culture demonstrated that elevated copper levels accelerated cell maturation, whereas decreased Cu levels, achieved by addition of the Cu chelator tetraethylenepentamine (TEPA), attenuated *ex vivo* human HSPC differentiation, resulting in expansion of early progenitor cells. TEPA-supplemented, long-term cytokine cultures of $CD34^+$ cord blood cells significantly increased the number of $CD34^+CD38^-$ cells and enhanced NOD/SCID repopulating capacity.[74,75] A phase I/II clinical trial in which TEPA-cultured cord blood cells were coinfused with uncultured cord blood cells showed the safety of this approach.[76] Although engraftment of TEPA-treated cells could be observed in almost all study subjects, the time required for neutrophil and platelet recovery was not changed compared with uncultured cord blood cells.[76] The efficiency of TEPA-cultured HSPC transplantation product is currently under investigation in an ongoing phase II/III study.

HDIs. Since stem cells are believed to be characterized by a specific (transcriptionally permissive) epigenetic status, epigenetically active compounds could possibly modulate stem cell fates. Several small molecules have been identified that alter the epigenetic status of cells. Histone deacetylase inhibitors (HDIs) or demethylating agents are example of such compounds. Several groups have studied the effects of valproic acid (VPA), an epigenetic drug known for its anticancer activity, on the regulation of *in vitro* HSC fate determination. Addition of VPA to cultures of murine or human HSPCs preserved the expression of primitive markers and an HSC phenotype.[77-79] VPA treatment was shown to maintain CFU-S activity in murine HSCs and resulted in elevated chimerism levels upon transplantation into lethally irradiated recipients.[77-80] Similar effects on the *ex vivo* expansion of human-mobilized peripheral blood $CD34^+$ HSCs were observed upon treatment with another HDI, chlamydocin.[81] Additionally, a combination of two epigenetic drugs was shown to additively affect HSC maintenance and/or expansion in culture.[82,83] Sequential addition of a demethylating agent, 5-aza- 2'-deoxycytidine methyltransferase (5azaD), and the HDI trichostatin A (TSA) to various cytokine cocktails has been shown to improve maintenance of stem cells in culture, compared with single compound treatment.[82,83] As expected, in these HSCs, HDI exposure increased histone acetylation, whereas 5aza decreased methylation.[82] HDI treatment has also been shown to result in up-regulation of cell-intrinsic HSPC fate regulators such as *HoxB4* and *AC133*, in activation of Wnt signaling, and in down-regulation of *p21*. Nevertheless, the exact mechanism of HDI-stimulated HSC expansion remains unclear.[77-83]

BIO. Small molecules that specifically target signaling pathways that play an important role in HSC self-renewal and maintenance could be beneficial for *ex vivo* expansion protocols. The synthetic compound 6-bromoindirubin-3'-oxime (BIO) can modulate Wnt signaling activity by targeting GSK3α and β, a negative regulator of Wnt signals. Stimulation of cord blood $CD34^+$ cells with BIO led to accumulation of slowly dividing cells and enhanced replating activity.[84,85]

Additionally, the exposure of cultured $CD34^+$ cells to BIO significantly increased engraftment and chimerism levels of the NOD/SCID repopulating cells.[49] As a GSK3β inhibitor, BIO treatment resulted in accumulation of β-catenin and its nuclear localization; however, the expression of Wnt target genes was not changed upon treatment.[84,85] Therefore, the key mechanism of BIO, and how it is responsible for enhanced HSPC activity, remain unknown.

SR1. Recent reports have shown that unbiased screens to search for factors that are able to maintain/expand HSCs can identify novel compounds with HSC self-renewal stimulatory activity. Using high-throughput screening, Boitano *et al*. identified an acryl hydrocarbon receptor (AhR) antagonist, referred to as StemRegenin1 (SR1) that was capable of enhancing human $CD34^+$ HSC cell amplification.[86] Cord blood $CD34^+$ cells cultured in the presence of SR1 led to a high net increase in the number of $CD34^+$ cells. Importantly, SR1-treated human cells demonstrated a 17-fold increase in cell numbers that were capable of hematopoietic reconstitution of sublethally irradiated mice and showed enhanced multilineage, long-term potential. Although it has been shown that hHSCs express AhR, which has been implicated in pathways regulating hematopoiesis, pathways including HES-1, β-catenin, and CXCR4, the exact mechanism of SR1-induced HSC expansion is not known yet.[86]

PGE_2. Another novel compound, the small lipid mediator prostaglandin E_2 (PGE_2), was identified as a regulator of HSC self-renewal using high-throughput library screens in zebrafish.[87] Chemicals enhancing PGE_2 synthesis have been shown to increase HSC numbers in zebrafish and mouse embryos, whereas those blocking its synthesis decrease stem cell numbers, indicating that modulation of the prostaglandin pathway might affect stem cell characteristics.[87] Components of the prostaglandin pathway and PGE_2 receptors are present on both mouse and human stromal cells and HSCs.[87,88] The enhancing role of PGE_2 on adult HSCs has been demonstrated in competitive transplantation models, where short stimulation of mouse cells with PGE_2 prior to transplantation increased the frequency of short- and long-term repopulating cells.[87] Hoggatt *et al*. confirmed the stimulatory effects of PGE_2 on mouse HSCs and additionally reported that the induced competitive advantage of treated HSCs remained upon serial transplantation. Although the exact mechanism of PGE_2 is not known, studies have shown that the PGE_2 effects might be explained by stimulation of HSC survival, proliferation, and self-renewal associated with upregulation of survivin, an inhibitor of apoptosis, and with a reduction of the active intercellular form of caspase-3. Furthermore, PGE_2 may enhance HSPC homing, as suggested by upregulated expression of the *CXCR4* gene.[88]

NR-101. The studies reported above have employed the use of chemical compounds added to cultures that also contain cytokines. Yet, small molecules have also been used as a full replacement of cytokines. Tpo is one of the most efficient cytokines supporting HSC proliferation and survival in culture and is widely used in HSC expansion protocols. Several small molecules have been chemically synthesized that are able to activate c-MPL, the receptor forTpo. Nishino *et al*. screened more than 400 of these molecules for their HSC stimulatory role and found a novel small molecule c-MPL agonist, NR-101 that more effectively expanded HSCs *ex vivo* compared with Tpo. Following culture, NR-101 increased $CD34^+$ and $CD34^+CD38^-$ cell numbers and NOD/SCID repopulating cell frequencies compared with Tpo or with fresh cord

blood CD34$^+$ cells. NR-101 was shown to activate the major pathways of Tpo/c-MPL signaling, but displayed extended signal activation compared to Tpo.[89]

Finding that HSPC fate can be modulated chemically by addition of small molecules to the culture media is convenient in light of *ex vivo* HSC expansion and offers, next to cytokines and developmental factors, an additional level of control of HSC self-renewal and differentiation *in vitro*. The promising potential of chemically enhanced HSC self-renewal and its cooperation with other self-renewal factors should be carefully evaluated and tested for use in clinical stem cell expansion protocols.

CONCLUSIONS

Although HSCs can expand extensively *in vivo*, conditions that reliably induce robust HSC expansion *in vitro* have yet to be discovered. Expansion attempts with the use of hematopoietic cytokines were rather disappointing and therefore diminished the initial enthusiasm of *ex vivo* stem cell expansion. A potential role of developmental factors and the discovery that self-renewal can be controlled by chemical compounds has revived this hope. Today, thousands of molecules are screened for their effects on HSC fates, and the utility of these factors for *in vitro* stem cell expansion is being investigated. Experience gained from many years of research indicates that the most comprehensive approach to develop optimal HSC expansion conditions may involve a combination of methods. It is likely that these conditions would involve multiple biological and chemical compounds that act in concert to induce cell survival and division while simultaneously preventing stem cell differentiation. Accordingly, several signaling pathways and/or compounds have been reported to act additively or synergistically to modulate HSPC fates in culture.[79,90,91] Additionally, methods that improve HSC homing and survival after transplantation could also be combined with *ex vivo* HSC expansion to potentially further enhance the efficiency of HSPC engraftment. Difficulties in defining HSC expansion conditions result partly from insufficient understanding of the molecular mechanisms controlling HSC fate determination. Identification of key molecules and the interaction networks important for self-renewal could significantly improve expansion attempts and may also lead to development of specific compounds targeting these self-renewal factors. Importantly, the challenge remains to assess to what extent it will be possible to generate fully functional HSCs, and their derivatives, for future clinical regenerative medicine applications.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Barker J. N. 2007. Umbilical Cord Blood (UCB) Transplantation: An Alternative to the Use of Unrelated Volunteer Donors? ASH Education Program Book. 2007: 55-61.
2. Wagner J. E., J. N. Barker, T. E. DeFor *et al*. 2002. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. *Blood*. 100: 1611-1618.
3. Brunstein C. G., J. A. Gutman, D. J. Weisdorf *et al*. 2010. Allogeneic hematopoietic cell transplantation for hematological malignancy: relative risks and benefits of double umbilical cord blood. *Blood*. 116: 4693–4699
4. Pawliuk R., C. Eaves & R. Humphries. 1996. Evidence of both ontogeny and transplant dose-regulated expansion of hematopoietic stem cells in vivo. *Blood*. 88: 2852-2858.
5. Iscove N. N. & K. Nawa. 1997. Hematopoietic stem cells expand during serial transplantation in vivo without apparent exhaustion. *Curr. Biol*. 7: 805-808.
6. Osawa M., K. Hanada, H. Hamada *et al*. 1996. Long-Term Lymphohematopoietic Reconstitution by a Single CD34-Low/Negative Hematopoietic Stem Cell. *Science*. 273: 242-245.
7. Zhu J. & S. G. Emerson. 2002. Hematopoietic cytokines, transcription factors and lineage commitment. *Oncogene*. 21: 3295-3313.
8. Akala O. O. & M. F. Clarke. 2006. Hematopoietic stem cell self-renewal. *Curr. Opin. Genet. Dev*. 16: 496-501.
9. Sauvageau G., U. Thorsteinsdottir, C. J. Eaves *et al*. 1995. Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. *Genes Dev*. 9: 1753-1765.
10. Antonchuk J., G. Sauvageau & R. K. Humphries. 2002. HOXB4-Induced Expansion of Adult Hematopoietic Stem Cells Ex Vivo. *Cell*. 109: 39-45.
11. Krosl J., P. Austin, N. Beslu *et al*. 2003. In vitro expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein. *Nat. Med*. 9: 1428-1432.
12. Fischbach N. A., S. Rozenfeld, W. Shen *et al*. 2005. HOXB6 overexpression in murine bone marrow immortalizes a myelomonocytic precursor in vitro and causes hematopoietic stem cell expansion and acute myeloid leukemia in vivo. *Blood*. 105: 1456-1466.
13. Lawrence H. J., J. Christensen, S. Fong *et al*. 2005. Loss of expression of the Hoxa-9 homeobox gene impairs the proliferation and repopulating ability of hematopoietic stem cells. *Blood*. 106: 3988-3994.
14. Thorsteinsdottir U., A. Mamo, E. Kroon *et al*. 2002. Overexpression of the myeloid leukemia–associatedHoxa9 gene in bone marrow cells induces stem cell expansion. *Blood*. 99: 121-129.
15. Fournier M., C. Lebert-Ghali, G. Krosl *et al*. 2012. HOXA4 Induces Expansion of Hematopoietic Stem Cells In Vitro and Confers Enhancement of Pro-B-Cells In Vivo. *Stem Cells and Dev*. 21: 133-142.
16. Kamminga L. M., L. V. Bystrykh, A. de Boer *et al*. 2006. The Polycomb group gene Ezh2 prevents hematopoietic stem cell exhaustion. *Blood*. 107: 2170-2179.
17. Iwama A., H. Oguro, M. Negishi *et al*. 2004. Enhanced Self-Renewal of Hematopoietic Stem Cells Mediated by the Polycomb Gene Product Bmi-1. *Immunity*. 21: 843-851.
18. Rizo A., B. Dontje, E. Vellenga *et al*. 2008. Long-term maintenance of human hematopoietic stem/progenitor cells by expression of BMI1. *Blood*. 111: 2621-2630.
19. Bartel D. P. 2004. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell*. 116: 281-297.
20. Bartel D. P. 2009. MicroRNAs: Target Recognition and Regulatory Functions. *Cell*. 136: 215-233.
21. Friedman R. C., K. K. Farh, C. B. Burge *et al*. 2009. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*. 19: 92-105.
22. Chen C., L. Li, H. F. Lodish *et al*. 2004. MicroRNAs Modulate Hematopoietic Lineage Differentiation. *Science*. 303: 83-86.
23. Arnold C. P., R. Tan, B. Zhou *et al*. 2011. MicroRNA programs in normal and aberrant stem and progenitor cells. *Genome Res*. 5: 798-810
24. Guo S., J. Lu, R. Schlanger *et al*. 2010. MicroRNA miR-125a controls hematopoietic stem cell number. *Proc. Natl. Acad. Sci*. 107: 14229-14234.
25. Raaijmakers M. H. G. P., S. Mukherjee, S. Guo *et al*. 2010. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature*. 464: 852-857.
26. O'Connell R. M., A. A. Chaudhuri, D. S. Rao *et al*. 2010. MicroRNAs enriched in hematopoietic stem cells differentially regulate long-term hematopoietic output. *Proc. Natl. Acad. Sci*. 107: 14235–40.
27. Ooi A. G. L., D. Sahoo, M. Adorno *et al*. 2010. MicroRNA-125b expands hematopoietic stem cells and enriches for the lymphoid-balanced and lymphoid-biased subsets. *Proc. Natl. Acad. Sci*. 107: 21505-21510.
28. Gerrits A., M. A. Walasek, S. Olthof *et al*. 2011. Genetic screen identifies microRNA cluster 99b/let-7e/125a as a regulator of primitive hematopoietic cells. *Blood*. 119: 377–87.
29. Bousquet M., M. H. Harris, B. Zhou *et al*. 2010. MicroRNA miR-125b causes leukemia. *Proc. Natl. Acad. Sci*. 107: 21558-21563.
30. Han Y., C. Y. Park, G. Bhagat *et al*. 2010. microRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia. *J Exp Med*. 207: 475-489.
31. Ogawa M. 1993. Differentiation and proliferation of hematopoietic stem cells. *Blood*. 81: 2844-2853.
32. Metcalf D. 2008. Hematopoietic cytokines. *Blood*. 111: 485-491.
33. Sauvageau G., N. N. Iscove & R. K. Humphries. 2004. In vitro and in vivo expansion of hematopoietic stem cells. *Oncogene*. 23: 7223-7232.
34. Goff J. P., D. S. Shields & J. S. Greenberger. 1998. Influence of Cytokines on the Growth Kinetics and Immunophenotype of Daughter Cells Resulting From the First Division of Single CD34+Thy-1+lin– Cells. *Blood*. 92: 4098-4107.
35. Glimm H. & C. J. Eaves. 1999. Direct Evidence for Multiple Self-Renewal Divisions of Human In Vivo Repopulating

Hematopoietic Cells in Short-Term Culture. *Blood*. 94: 2161-2168.

36. Nakauchi H., Sudo K., & Ema H. 2001. Quantitative Assessment of the Stem Cell Self-Renewal Capacity. *Ann. N. Y. Acad. Sci*. 938: 18-25.
37. Uchida N., B. Dykstra, K. J. Lyons *et al*. 2003. Different in vivo repopulating activities of purified hematopoietic stem cells before and after being stimulated to divide in vitro with the same kinetics. *Exp. Hematol*. 31: 1338-1347.
38. Ema H., H. Takano, K. Sudo *et al*. 2000. In Vitro Self-Renewal Division of Hematopoietic Stem Cells. *J Exp Med*. 192: 1281-1288.
39. Audet J., C. L. Miller, C. J. Eaves *et al*. 2002. Common and distinct features of cytokine effects on hematopoietic stem and progenitor cells revealed by dose-response surface analysis. *Biotechnol. Bioeng.* 80: 393-404.
40. Miller C. L. & C. J. Eaves. 1997. Expansion in vitro of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability. *Proc. Natl. Acad. Sci*. 94: 13648-13653.
41. Bhatia M., D. Bonnet, U. Kapp *et al*. 1997. Quantitative Analysis Reveals Expansion of Human Hematopoietic Repopulating Cells After Short-term Ex Vivo Culture. *J Exp Med*. 186: 619-624.
42. de Lau W., N. Barker, T. Y. Low *et al*. 2011. Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature*. 476: 293-297.
43. Reya T., A. W. Duncan, L. Ailles *et al*. 2003. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature*. 423: 409-414.
44. Austin T. W., G. P. Solar, F. C. Ziegler *et al*. 1997. A Role for the Wnt Gene Family in Hematopoiesis: Expansion of Multilineage Progenitor Cells. *Blood*. 89: 3624-3635.
45. Willert K., J. D. Brown, E. Danenberg *et al*. 2003. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature*. 423: 448-452.
46. Murdoch B., K. Chadwick, M. Martin *et al*. 2003. Wnt-5A augments repopulating capacity and primitive hematopoietic development of human blood stem cells in vivo. *Proc. Natl. Acad. Sci*. 100: 3422-3427.
47. Van Den Berg D. J., A. K. Sharma, E. Bruno *et al*. 1998. Role of Members of the Wnt Gene Family in Human Hematopoiesis. *Blood*. 92: 3189-3202.
48. Luis T., B. E. Naber, P. C. Roozen *et al*. 2011. Canonical Wnt Signaling Regulates Hematopoiesis in a Dosage-Dependent Fashion. *Cell Stem Cell*. 9: 345-356.
49. MacDonald B. T., K. Tamai & X. He. 2009. Wnt/β-Catenin Signaling: Components, Mechanisms, and Diseases. *Dev Cell*. 17: 9-26.
50. Duncan A. W., F. M. Rattis, L. N. DiMascio *et al*. 2005. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nat. Immunol*. 6: 314-322.
51. Varnum-Finney B., L. E. Purton, M. Yu *et al*. 1998. The Notch Ligand, Jagged-1, Influences the Development of Primitive Hematopoietic Precursor Cells. *Blood*. 91: 4084-4091.
52. Varnum-Finney B., C. Brashem-Stein & I. D. Bernstein. 2003. Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability. *Blood*. 101: 1784-1789.
53. Delaney C., B. Varnum-Finney, K. Aoyama *et al*. 2005. Dose-dependent effects of the Notch ligand Delta1 on ex vivo differentiation and in vivo marrow repopulating ability of cord blood cells. *Blood*. 106: 2693-2699.
54. Delaney C., S. Heimfeld, C. Brashem-Stein *et al*. 2010. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. *Nat. Med*. 16: 232-236.
55. Bhardwaj G., B. Murdoch, D. Wu *et al*. 2001. Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat. Immunol*. 2: 172-180.
56. Bhatia M., D. Bonnet, D. Wu *et al*. 1999. Bone Morphogenetic Proteins Regulate the Developmental Program of Human Hematopoietic Stem Cells. *J Exp Med*. 189: 1139-1148.
57. de Haan G., E. Weersing, B. Dontje *et al*. 2003. In Vitro Generation of Long-Term Repopulating Hematopoietic Stem Cells by Fibroblast Growth Factor-1. *Dev Cell*. 4: 241-251.
58. Yeoh J. S. G., R. van Os, E. Weersing *et al*. 2006. Fibroblast Growth Factor-1 and -2 Preserve Long-Term Repopulating Ability of Hematopoietic Stem Cells in Serum-Free Cultures. *Stem Cells*. 24: 1564-1572.
59. Zhang C. C. & H. F. Lodish. 2004. Insulin-like growth factor 2 expressed in a novel fetal liver cell population is a growth factor for hematopoietic stem cells. *Blood*. 103: 2513-2521.
60. Huynh H., S. Iizuka, M. Kaba *et al*. 2008. Insulin-Like Growth Factor-Binding Protein 2 Secreted by a Tumorigenic Cell Line Supports Ex Vivo Expansion of Mouse Hematopoietic Stem Cells. *Stem Cells*. 26: 1628-1635.
61. Zhang C. C., M. Kaba, G. Ge *et al*. 2006. Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. *Nat. Med*. 12: 240-245.
62. Zhang C. C., M. Kaba, S. Iizuka *et al*. 2008. Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. *Blood*. 111: 3415-3423.
63. Himburg H. A., G. G. Muramoto, P. Daher *et al*. 2010. Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells. *Nat. Med*. 16: 475-482.
64. Istvanffy R., M. Kröger, C. Eckl *et al*. 2011. Stromal pleiotrophin regulates repopulation behavior of hematopoietic stem cells. *Blood*. 118: 2712-2722.
65. Huangfu D., R. Maehr, W. Guo *et al*. 2008. Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat Biotech*. 26: 795-797.
66. Li W. & S. Ding. 2010. Small molecules that modulate embryonic stem cell fate and somatic cell reprogramming. *Trends Pharmacol*. Sci. 31: 36-45.
67. Schugar R. C., P. D. Robbins & B. M. Deasy. 2007. Small molecules in stem cell self-renewal and differentiation. *Gene Ther*. 15: 126-135.
68. Collins S. J. 2002. The role of retinoids and retinoic acid receptors in normal hematopoiesis. *Leukemia*. 16: 1896-1905.
69. Degos L. & Z. Y. and Wang. 2001. All *trans* retinoic acid in acute promyelocytic leukemia. *Oncogene*. 20: 7140-7145.
70. Purton L. E., S. Dworkin, G. H. Olsen *et al*. 2006. RARγ is critical for maintaining a balance between

hematopoietic stem cell self-renewal and differentiation. *J Exp Med*. 203: 1283-1293.

71. Purton L. E., I. D. Bernstein & S. J. Collins. 1999. All-Trans Retinoic Acid Delays the Differentiation of Primitive Hematopoietic Precursors (lin−c-kit +Sca-1+) While Enhancing the Terminal Maturation of Committed Granulocyte/Monocyte Progenitors. *Blood*. 94: 483-495.
72. Purton L. E., I. D. Bernstein & S. J. Collins. 2000. All-trans retinoic acid enhances the long-term repopulating activity of cultured hematopoietic stem cells. *Blood*. 95: 470-477.
73. Chute, J.P., G.G. Muramoto, J. Whitesides, *et al*. 2006. Inhibition of aldehyde dehydrogenase and retinoid signaling induces the expansion of human hematopoietic stem cells. *Proc. Natl. Acad. Sci.* 103: 11707–11712.
74. Peled, T., E. Landau, J.Mandel, *et al*. 2004. Linear polyamine copper chelator tetraethylenepentamine augments long-term ex vivo expansion of cord blood-derived CD34+ cells and increases their engraftment potential in NOD/SCID mice. *Exp. Hematol.* 32: 547–555
75. Peled, T., E. Glukhman, N. Hasson, *et al*. 2005. Chelatable cellular copper modulates differentiation and self-renewal of cord blood-derived hematopoietic progenitor cells. *Exp. Hematol.* 33: 1092–1100.
76. Peled, T., J.Mandel, R.N. Goudsmid, *et al*. 2004. Pre-clinical development of cord blood-derived progenitor cell graft expanded ex vivo with cytokines and the polyamine copper chelator tetraethylenepentamine. *Cytotherapy* 6: 344– 355.
77. Bug G., H. Gül, K. Schwarz *et al*. 2005. Valproic Acid Stimulates Proliferation and Self-renewal of Hematopoietic Stem Cells. *Cancer Res*. 65: 2537-2541.
78. De Felice L., C. Tatarelli, M. G. Mascolo *et al*. 2005. Histone Deacetylase Inhibitor Valproic Acid Enhances the Cytokine-Induced Expansion of Human Hematopoietic Stem Cells. *Cancer Res*. 65: 1505-1513.
79. Walasek M., A., L. Bystrykh, V. van den Boom *et al*. 2012. The combination of valproic acid and lithium delay hematopoietic stem/progenitor cell differentiation. *Blood*. 119: 3050-9
80. Seet L., E. Teng, Y. Lai *et al*. 2009. Valproic acid enhances the engraftability of human umbilical cord blood hematopoietic stem cells expanded under serum-free conditions. *Eur. J. Haematol.* 82: 124-132.
81. Young J. C., S. Wu, G. Hansteen *et al*. 2004. Inhibitors of histone deacetylases promote hematopoietic stem cell self-renewal. *Cytotherapy*. 6: 328-336.
82. Milhem M., N. Mahmud, D. Lavelle *et al*. 2004. Modification of hematopoietic stem cell fate by 5aza 2'deoxycytidine and trichostatin A. *Blood*. 103: 4102-4110.
83. Araki H., K. Yoshinaga, P. Boccuni *et al*. 2007. Chromatin-modifying agents permit human hematopoietic stem cells to undergo multiple cell divisions while retaining their repopulating potential. *Blood*. 109: 3570-3578.
84. Holmes T., T. A. O'Brien, R. Knight *et al*. 2008. Glycogen Synthase Kinase-3? Inhibition Preserves Hematopoietic Stem Cell Activity and Inhibits Leukemic Cell Growth. *Stem Cells*. 26: 1288-1297.
85. Ko K., T. Holmes, P. Palladinetti *et al*. 2011. GSK-3β Inhibition Promotes Engraftment of Ex Vivo-Expanded Hematopoietic Stem Cells and Modulates Gene Expression. *Stem Cells*. 29: 108-118.
86. Boitano A. E., J. Wang, R. Romeo *et al*. 2010. Aryl Hydrocarbon Receptor Antagonists Promote the Expansion of Human Hematopoietic Stem Cells. *Science*. 329: 1345-1348.
87. North T. E., W. Goessling, C. R. Walkley *et al*. 2007. Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature*. 447: 1007-1011.
88. Hoggatt J., P. Singh, J. Sampath *et al*. 2009. Prostaglandin E2 enhances hematopoietic stem cell homing, survival, and proliferation. *Blood*. 113: 5444-5455.
89. Nishino T., K. Miyaji, N. Ishiwata *et al*. 2009. Ex vivo expansion of human hematopoietic stem cells by a small-molecule agonist of c-MPL. *Exp. Hem*. 37: 1364-1377.
90. Watts K. L., C. Delaney, R. K. Humphries *et al*. 2010. Combination of HOXB4 and Delta-1 ligand improves expansion of cord blood cells. *Blood*. 116: 5859–66.
91. Perry J. M., X. C. He, R. Sugimura *et al*. 2011. Cooperation between both Wnt/β-catenin and PTEN/PI3K/Akt signaling promotes primitive hematopoietic stem cell self-renewal and expansion. *Genes Dev*. 25: 1928-1942.

CHAPTER 3

GENETIC SCREENS IDENTIFIES MICRORNA CLUSTER 99B/LET7E/125A AS A REGULATOR OF PRIMITIVE HEMATOPOIETIC CELLS

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ABSTRACT

Hematopoietic stem/progenitor cell (HSPC) traits differ between genetically distinct mouse strains. For example, DBA/2 mice have a higher HSPC frequency compared with C57BL/6 mice. We performed a genetic screen for microRNAs that are differentially expressed between LSK, $LS^{-}K^{+}$, erythroid and myeloid cells isolated from C57BL/6 and DBA/2 mice. This analysis identified 131 microRNAs that were differentially expressed between cell types and 15 that were differentially expressed between mouse strains. Of special interest was an evolutionary conserved miR cluster located on chromosome 17 consisting of miR-99b, let-7e, and miR-125a. All cluster members were most highly expressed in LSKs and down-regulated upon differentiation. In addition, these microRNAs were higher expressed in DBA/2 cells compared with C57BL/6 cells, and thus correlated with HSPC frequency. To functionally characterize these microRNAs, we overexpressed the entire miR-cluster 99b/ let-7e/125a and miR-125a alone in BM cells from C57BL/6 mice. Overexpression of the miR-cluster or miR-125a dramatically increased day-35 CAFC activity and caused severe hematopoietic phenotypes upon transplantation. We showed that a single member of the miR-cluster, namely miR-125a, is responsible for the majority of the observed miR-cluster overexpression effects. Finally, we performed genome-wide gene expression arrays and identified candidate target genes through which miR-125a may modulate HSPC fate.

INTRODUCTION

HSCs sustain lifelong blood production, and are characterized by their self-renewal capacity and multilineage differentiation potential. HSCs give rise to progenitors that differentiate into the various blood cell types. Many characteristics of the hematopoietic stem and progenitor cell (HSPC) compartment display mouse strain-dependent genetic variation. For example, compared with C57BL/6 (B6) mice, DBA/2 (D2) mice have a higher HSPC frequency and cycling rate.[1-4] The variation between these inbred mouse strains can be exploited to identify potential modulators of such HSPC traits. Useful in this respect has been the generation of BXD recombinant inbred mouse strains.[5,6] Each of these strains represents a unique, but stable, genetic mosaic of B6 and D2 alleles. By making use of these strains, variation in many different HSPC traits has been mapped to genomic regions (reviewed in Gerrits et al[7]). Previously, we analyzed mRNA expression variation in developmentally related cell types isolated from the BXD mouse panel.[8,9] In those studies, we identified groups of genes that were differentially expressed across mouse strains and therefore potentially causal for the variation in HSPC traits.

MicroRNAs are evolutionary conserved small (~22-nucleotide) noncoding RNAs that fine-tune gene expression by base-pairing with target mRNAs, leading to mRNA destabilization or translational repression.[10-12] Each microRNA can coordinately target hundreds of different mRNAs,[13] and each mRNA can harbor multiple microRNA target sites, creating complex microRNA/mRNA regulatory circuitries. A growing body of evidence has implicated specific microRNAs in the regulation of HSPC fate. For example, miR-181, miR-150, and the miR-17~92 cluster have proven to be essential for lymphoid development,[14-18] miR-223 for myeloid development,[19,20] and miR-155 for both lymphoid and myeloid development.[21-23] Dysregulated expression of these and other microRNAs has been shown to contribute to the pathogenesis and progression of hematologic malignancies.[24,25]

To determine whether the variation in gene expression and HSPC traits across mouse strains could be due to natural variation in microRNA expression, we initiated a genome-wide microRNA expression study of $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK), $Lin^-Sca\text{-}1^-c\text{-}Kit^+$ (LS^-K^+), erythroid and myeloid cells isolated from B6 and D2 mice. We identified both cell type-dependent and mouse strain-dependent microRNAs. Interestingly, we discovered an evolutionary conserved cluster of microRNAs (99b/let-7e/125a) that is most highly expressed in HSPCs and that is differentially expressed between mouse strains. To assess whether the differential expression of members of this cluster could be functional, we overexpressed both the entire miR-cluster 99b/let-7e/125a and miR-125a alone in HSPCs, and found that this conferred an initial competitive advantage to these cells. However, we also found that mice reconstituted with these cells developed myeloproliferative neoplasms (MPNs). Our data suggest that miR-125a is responsible for the majority of the hematopoietic effects observed for the entire miR-cluster 99b/let-7e/125a. Finally, we identified the candidate functional downstream targets through which miR-125a may be able to modulate HSPC fate.

MATERIALS AND METHODS

Mice. Female B6 (CD45.2) and D2 mice were purchased from Harlan and housed under clean conventional conditions. Female and male B6.SJL (CD45.1) mice were bred at the Central Animal Facility of the University of Groningen. All animal experiments were approved by the Groningen University Animal Care Committee.

Cell purification. BM cells were flushed from the femurs and tibias of 3 to 5 mice and pooled. Nucleated cells were stained with a panel of Alexa Fluor 700 (A700)-labeled lineage-specific Abs, FITC-labeled anti-Sca-1, PE-labeled anti-c-Kit, PE-cyanine-7 (PE-Cy7)-labeled anti–TER-119, and allophycocyanin- labeled anti–Gr-1. Abs were purchased from BioLegend. Triplicates were generated for each of the 8 conditions (4 cell types, 2 mouse strains).

MicroRNA expression analysis. Total RNA was isolated using the miRNeasy Mini Kit (QIAGEN) in accordance with the manufacturer's protocol. Total RNA (100 ng) was labeled and hybridized to Agilent 8X15K Mouse miRNA arrays. Labeling, hybridization, and washing were performed by Oxford Gene Technology using the Agilent Mouse microRNA Microarray Kit. Data were extracted using Agilent Feature Extraction Software (Version 9.5.3.1). Data were thresholded at 1, log2-transformed, and normalized to the 75th percentile using GeneSpring GX11.0 (Agilent). On the basis of principal component analysis, 1 sample was removed from further analysis. The starting dataset represented 577 noncontrol probes. False positives were excluded by only pursuing with those probes that were flagged as present in at least 2 of 3 replicates in any 1 of 8 conditions. The quality-filtered expression values were median-centered per microRNA and clustered (Euclidean distance, complete linkage) using Genesis.[26] Two-way ANOVA was applied to identify cell type-dependent and mouse strain-dependent microRNAs. A Benjamini-Hochberg false discovery rate correction was applied to control for multiple testing (P<.05). All microarray data are available for viewing on the Gene Expression Omnibus (GEO) under accession number GSE33691.

Retroviral vectors. Genomic DNA was isolated from B6 cells and a 1.2-kb region spanning the miR-99b, let-7e, and miR-125a stem loop sequences was amplified (forward primer: 5'-CCTCGA GTGGACTGAGGAGAATTGAGTGCAAG-3', reverse primer: 5'-GCAATTGTGCCCTGAAGATCAG CAGGAAC-3'; Biolegio). The resulting PCR product was extracted from gel, TOPO-cloned into PCR4, cut from PCR4 using *Xho*I and *Mun*I, and subcloned into the *Xho*I-*Eco*RI site of the MXW-pPGK-IRES-EGFP vector, which is a murine stem cell virus-based vector with constitutively active PolII type promoter.[27] The miR-125a vector was constructed from the miR-99b/let-7e/125a vector by deleting the *Eco*RI-*Bst*BI fragment (carrying the miR-99b/let7e segment of the miR cluster), followed by vector religation with an aliquot of random hexamers. The miR-155 gene product and the MXW-pPGK-IRES-EGFP vector were a kind gift from Prof Chen (Stanford University School of Medicine, Stanford, CA). The miR-cluster 99b/let-7e/125a, miR-125a and miR-155 fragments were sequence verified (StarSeq; see supplemental Methods).

Retroviral overexpression of microRNAs in primary BM cells. Primary BM cells were isolated from B6 mice 4 days after IP injection of 150 mg/kg 5-fluorouracil (Pharmachemie Haarlem), and cultured in StemSpan (StemCell Technologies) supplemented with 10% FCS, 300 ng/mL

recombinant mouse SCF (rmSCF; Peprotech), 20 ng/mL rmIL11 (R&D Systems), 1 ng/mL Flt3 ligand (Amgen), penicillin, and streptomycin. BM cells were transduced by transfecting Phoenix ecotropic packaging cells with 1-2 µg of pDNA (MXW empty vector, MXW-miR-cluster 99b/let-7e/125a, MXW-miR-125a, and MXW-miR-155) and 3-6µL of Fugene HD (Roche). Virus-containing supernatant harvested 48 and 72 hours later was used to transduce 4-6x10^5 BM cells per 3.5-cm well. Three independent transductions were performed per condition per experiment. Five days after the first transduction, viable (negative for propidium iodide) EGFP$^+$ cells were collected and tested in *in vitro* assays and collected in RNA lysis buffer (QIAGEN) for gene expression studies. Nonsorted cells were tested in an *in vivo* BM transplantation setting.

Quantitative PCR validation. We converted selected microRNAs to cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Next, real-time PCR with TaqMan MicroRNA assays (Applied Biosystems) was performed using the iCycler system (Bio-Rad). The following assays were used: hsa-miR-99b, hsa-let-7e, hsa-miR-125a-5p, mmu-miR-155, and snoRNA202. The comparative delta-delta Ct approximation method was used to analyze relative changes in gene expression.[28] Each of the 3 samples was analyzed in triplicate and normalized to the endogenous control snoRNA202.

CAFC assays. The cobblestone-area forming cell (CAFC) assay was performed as previously described.[29,30]

Primary BM transplantation. BM cells were transplanted into lethally irradiated recipients without prior sorting for EGFP expression. Two independent transplantation experiments were performed, where B6 and B6.SJL mice were exchangeably used as a source of donor cells or transplant recipients. In both experiments, 9 mice (3 independent transductions x 3 mice) received transplants per condition with 5-10x10^6 cells each. On a 4- to 6-week basis posttransplantation blood analyses were performed. Blood cell numbers were counted using a Medonic hematology analyzer (Boule Medical). Subsequently, cells were stained with PE-labeled anti-CD45.2, Pacific Blue-labeled anti-CD45.1, allophycocyanin-Cy7–labeled anti–Gr-1, PE-Cy7-labeled anti-CD11b, Pacific Orange-labeled anti-CD45R/B220 (Invitrogen, Caltag Laboratories), and allophycocyanin-labeled anti-CD3ε. Abs were purchased from BioLegend, unless otherwise specified. Data were acquired using an LSR II (BD Biosciences) and analyzed using FlowJo software (TreeStar). At the time of sacrifice, the peripheral blood, BM, spleen, liver, and in a few instances, lung tissues were analyzed. Cell counts, cytospin preparations, and FACS stainings were performed. Cells were stained using the aforementioned Abs mix or stained with lineage cocktail (A700-labeled anti-Mac1, A700-labeled anti-Gr-1, A700-labeled anti-TER-119, A700-labeled anti-CD3ε, A700-labeled anti-B220), PE-labeled anti-cKit, Pacific Blue-labeled anti-Sca-1, PE-Cy7-labeled anti-CD150 and A647-labeled anti-CD48. Cytospins were stained with May-Grunwald-Giemsa (MGG).

Secondary BM transplantation. EGFP$^+$CD45.2$^+$ BM cells from the primary miR cluster and empty vector mice received transplants in competition with freshly isolated wild-type CD45.1$^+$ BM cells in lethally irradiated recipients (CD45.2) at 4:1 (test to freshly isolated) ratios. For each of the conditions, 15 mice (3 individual primary mice x 5 secondary mice) received transplants with a total of 5x10^6 cells each. Blood analyses were performed on a 4- to 6-week basis posttransplantation.

Downstream target analysis. Genome-wide gene expression was profiled in BM cells transduced with empty vector, miR-cluster 99b/let-7e/125a, miR-125a, and miR-155. Heterogeneous populations of viable EGFP$^+$ BM cells were FACS-purified without any additional marker selection at 5 days after the first transduction (the exact time point at which the aforementioned *in vitro* and *in vivo* assays were initiated). All samples were analyzed in triplicate. Total RNA was isolated using the RNeasy Mini Kit (Qiagen). RNA was amplified using the Illumina TotalPrep RNA Amplification Kit (Ambion/Applied Biosystems) and hybridized to Sentrix MouseWG-6 Version 2.0 expression beadchips (Illumina) according to the manufacturer's instructions. Hybridization and washing were performed by our in-house Genome Analysis Facility. Scanning was carried out on the iScan System (Illumina). Data were extracted using GenomeStudio software (Illumina), without normalization or background subtraction. Data were thresholded at 1, log2- transformed, and quantile normalized using GeneSpring GX11.0 (Agilent). The starting dataset represented 45.281 probes. False positives were excluded by only pursuing with those probes that were flagged as marginal or present in all replicates in any 1 of 4 conditions (default detection *P* values cutoff 0.8 for present and 0.6 for absent were used for flags). Predicted (conserved) downstream microRNA targets were imported from the TargetScan database.[31]

URLs. All raw data were deposited in the NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/, accession number GSE33691). All processed microRNA expression data on the 4 cell types from B6 and D2 mice were deposited on HemDb (http://hemdb.org/).

RESULTS

Identification of cell type-dependent microRNA signatures. To identify cell type-dependent and mouse strain-dependent microRNAs in the hematopoietic system, we started a microarray-based microRNA profiling study of 4 developmentally related hematopoietic cell types isolated from the BM of B6 and D2 mice. We hybridized total RNA isolated from purified LSK multilineage cells, committed LS$^-$K$^+$ cells, erythroid TER-119$^+$ cells, and myeloid Gr-1$^+$ cells (Figure 1A) to Agilent microRNA arrays and evaluated microRNA expression levels. In total, we analyzed 23 samples representing 4 different cell types and 2 different mouse strains. Of the 577 microRNAs profiled, 147 were expressed in at least 1 of these 8 conditions. To study the relationship between the samples, as well as the underlying patterns of microRNA expression, we applied an unsupervised 2-way hierarchical clustering method using the 147 quality-filtered microRNAs. This analysis showed that the cell-type effect greatly exceeded the mouse strain effect (Figure 1B top tree), and revealed 8 distinct microRNA signatures (Figure 1B left tree; supplemental Table 1). Of special interest is signature I consisting of microRNAs that are most highly expressed in LSKs and down-regulated upon differentiation. Signature I consists of miR-125a-5p, miR-125b-5p, miR-126-3p, miR-130a, miR-155, miR-181d, miR-196b, miR-203, miR-222, miR-31, miR-99a, miR-99b, and let-7e. Considering their expression pattern, these microRNAs may represent important factors that keep cells from leaving the stem cell state. Signature II consists of microRNAs that are most highly expressed in LS$^-$K$^+$ cells, signatures III and IV are specific to erythroid cells, and signatures VI and VIII to myeloid cells. Signature VII consists of microRNAs that are expressed in all cell types except for LSKs, and may therefore consist

of microRNAs that are important for lineage specification, commitment, and differentiation. Finally, signature V does not show a clear expression preference. We confirmed well-known cell-type-specific microRNAs: eg miR-196b in signature I,[32] miR-451 in erythroid signature III,[33] and miR-223 in myeloid signature VIII.[19,20] The entire microRNA expression dataset is available online (supplemental Table 1 and in GEO [accession number GSE33690]), and queries for specific micro-RNAs can be done on www.hemdb.org.

Identification of mouse strain-dependent miR-cluster 99b/let-7e/125a. An analysis of variance (2-way ANOVA, corrected $P<.05$) yielded 131 differentially expressed microRNAs, all of which showed a cell-type effect and of which 15 also showed a mouse strain effect (Figure 1C, supplemental Table 1). In total, 4 of the 15 mouse strain-dependent microRNAs were present in signature I, namely miR-125a, miR-125b, miR-130a, and miR-99b. They were all most highly expressed in the primitive cell compartment and down-regulated upon differentiation and generally higher expressed in D2 cells compared with B6 cells. These 2 criteria qualify those microRNAs as potential candidates regulating HSPC traits. Interestingly however, 2 of these 4, miR-125a and miR-99b are part of an evolutionary conserved microRNA cluster that is localized on chromosome 17: miR-cluster 99b/let-7e/125a (Figure 1D). The third member of this cluster, let-7e, shows an expression pattern comparable with the other 2 members (Figure 1E), but its strain effect did not reach statistical significance (corrected $P=.064$). In addition, miR-125b is present in 2 paralogous microRNA clusters that are located on chromosomes 9 and 16 (Figure 1F). The chromosome 9 cluster consists of miR-100, let-7a, and miR-125b-1 and the chromosome 16 cluster consists of miR-99a, let-7c, and miR-125b-2. Although not all microRNA cluster members display a significant mouse strain effect, they all follow the same expression trend across cell types and mouse strains (Figure 1G). This view corroborates previous observations that proximal pairs of microRNAs (separated by < 50 kb) are generally coexpressed across tissues.[34]

Overexpression of miR-cluster 99b/let-7e/125a or miR-125a alone retains HSPCs in a primitive state. To assess whether differential expression of members of the 99b/let-7e/125a microRNA cluster could have functional consequences for HSPCs, and could be causal for the phenotypic differences between HSPCs from B6 and D2 mice, we performed gain-of-function studies. We cloned the entire 99b/let-7e/125a cluster, as these 3 microRNAs showed coordinated expression, and miR-125a alone in a retroviral vector (both in its natural genomic context) (Figure 2A). MiR-155, known to affect HSPC characteristics, was included as a positive control.[21] The miR-cluster 99b/let-7e/125a, miR-125a, and miR-155 were overexpressed in BM cells from 5-FU–treated mice. Transduced cells were sorted and overexpression of the individual members of miR-cluster 99b/let-7e/125a and miR-155 was validated using quantitative PCR (Figure 2B). Engineered expression levels of miR-cluster 99b/let-7e/125a members were substantially increased (1000-, 2-, 1500-fold, respectively) compared with the natural situation, in which members of the miR-cluster are on average 2-fold higher expressed in D2 than in B6 LSK cells (microarrays and quantitative PCR; data not shown). Concurrently, transduced $EGFP^+$ cells were used to initiate CAFC, a surrogate *in vitro* assay to quantify the number of HSPCs. In this assay, early appearing cobblestones (day 7) are considered to represent progenitors and late-appearing cobblestones (day 35) are considered to represent stem cells.[29,30] Day-7 CAFC activity

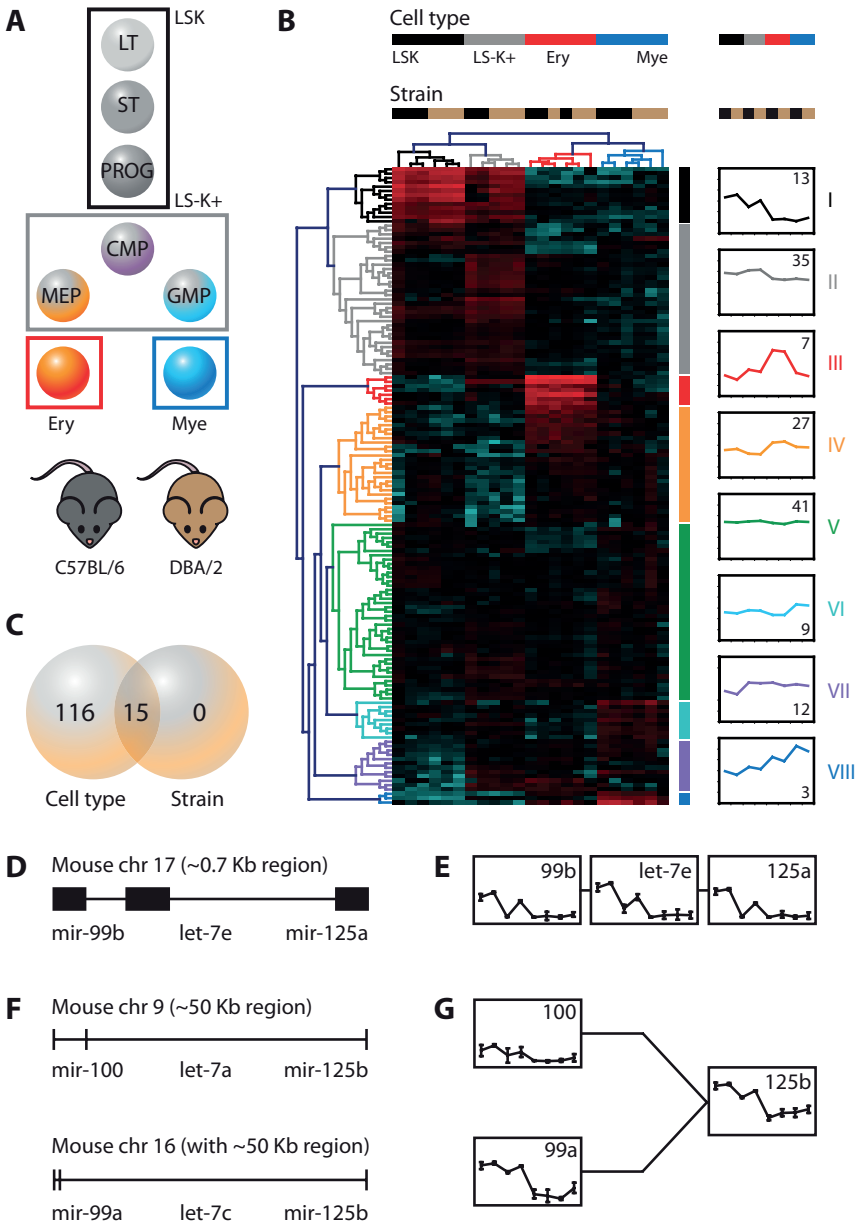

Figure 1. Genetic screen identifies cell type- and mouse strain-dependent microRNAs. (A) MicroRNA expression was evaluated in 4 developmentally related cell types isolated from the BM of C57BL/6 and DBA/2 mouse strains. (B) Hierarchical clustering was performed using the 147 quality-filtered probes (Euclidean distance, complete linkage). Samples are in columns, microRNAs in rows. For each probe, data were median-centered, with the lowest and highest intensity values in blue and red, respectively. Of each of the 8 microRNA signatures the average expression across cell types and mouse strains is shown (y-axis represents log2 expression ranging from 4 to 8). The number in the graphs indicates the number of microRNAs per signature. (C) Venn diagram showing the number of differentially expressed microRNAs between cell types and mouse strains. (D) An evolutionary conserved microRNA cluster, consisting of ▶

showed no differences between the control and microRNA-overexpressing cells. However, at day 35, a striking difference in the number of CAFCs was observed. The miR-155-overexpressing cells gave ~15-fold more CAFCs, whereas the miR-cluster 99b/let-7e/125a-overexpressing cells yielded a striking ~6.500-fold more CAFCs (Figure 2C left). Direct comparison between miR-cluster 99b/let-7e/125a and miR-125a alone revealed very similar CAFC activity profiles and no significant differences were observed between these 2 overexpression conditions. Both the miR-cluster 99b/let-7e/125a and miR-125a strongly increased day-35 CAFC activity (Figure 2C right). Moreover, for both the miR-cluster and miR-125a, a substantial amount of CAFC activity remained even up until day 70 (data not shown).

To summarize, BM cells overexpressing miR-cluster 99b/let-7e/125a, miR-125a, or miR-155 showed increased stem cell activity as measured by day-35 CAFC activity. These data suggest that overexpression of miR-cluster 99b/let-7e/125a or miR-125a alone retains cells in a primitive state.

Sustained expression of miR-cluster 99b/let-7e/125a or miR-125a in HSPCs provides a competitive advantage, but also results in MPNs. We next assayed the microRNA-overexpressing cells in a long-term competitive repopulation experiment *in vivo*. To achieve this, miR-cluster 99b/let-7e/125a, miR-125a alone and miR-155 were overexpressed in BM cells derived from 5-FU-treated mice and nonsorted cells were transplanted into lethally irradiated recipients. At 10 weeks posttransplantation, blood cell counts revealed a significant decrease in RBC numbers and a slight decrease in white blood cell (WBC) numbers for mice transplanted with miR-cluster 99b/let-7e/125a or miR-125a–overexpressing cells. However, whereas platelet counts were significantly decreased in miR-cluster reconstituted mice, this was not observed for miR-125a reconstituted mice. Recipients transplanted with miR-155 transduced cells showed a less dramatic decrease of RBC counts with no effect on WBC and platelet counts (Figure 3A). At this point in time, chimerism was assessed by quantifying the percentage of $EGFP^+$ cells within the donor fraction. Whereas within 10 weeks the chimerism levels of the control mice remained relatively stable at 36%, those of the miR-155 mice increased from 15% to 45%, and those of the miR-cluster 99b/let-7e/125a and miR-125a mice increased from 25% or 10%, respectively, to 90%. This indicates that in the latter 3 cases the microRNA-transduced cells had a competitive advantage over the nontransduced cells (Figure 3B). Detailed FACS analyses revealed that the $EGFP^+$ cells in both the miR-cluster 99b/let-7e/125a and miR-125a mice were enriched for granulocytes/macrophages (defined as being $Gr\text{-}1^+$ and/or $Mac\text{-}1^+$) at the expense of the number of B lymphocytes ($B220^+$; Figure 3C). Clearly, hematopoiesis was disturbed in

▶ miR-99b, let-7e, and miR-125a, located on mouse chromosome 17. (E) Expression of miR-cluster 99b/let-7e/125a members across cell types and mouse strains (axes: as in panel B). Shown is the mean± SD. (F) Paralogous microRNA clusters on chromosomes 9 and 16. (G) Expression of miR-100, miR-99a, and miR-125b across cell types and mouse strains (axes: as in panel B). For miR-125b only the cumulative expression of the chromosome 9 and 16 cluster could be assessed. Expression of let-7a and let-7c is not shown, as only the cumulative expression from multiple different genomic locations (including other chromosomes than 9, 16, and 17) could be assessed. Shown is the mean± SD. LT indicates long-term; ST, short-term; CMP, common myeloid progenitor; MEP, megakaryocyte-erythrocyte progenitor; and GMP, granulocyte-macrophage progenitor.

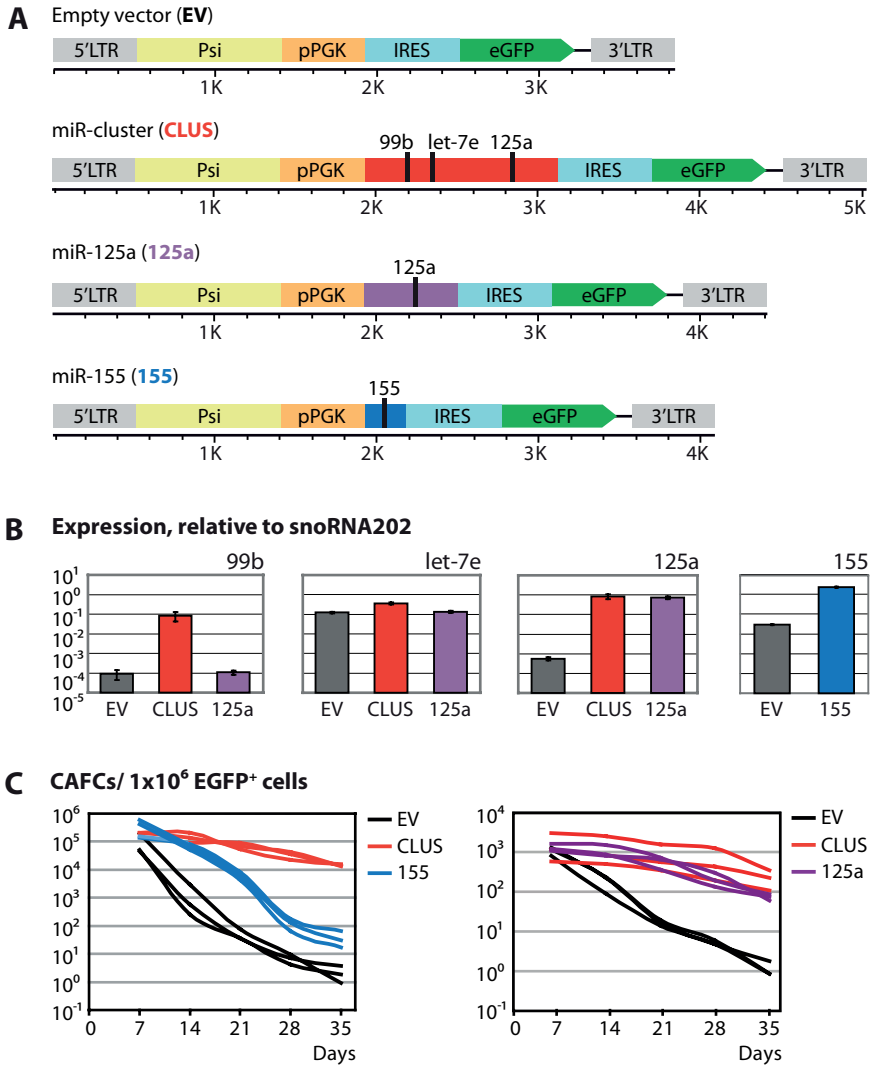

Figure 2. Overexpression of miR-cluster 99b/let-7e/125a or miR-125a alone fixes HSPCs in a primitive state. (A) Schematic presentation of the retroviral vectors used to overexpress miR-cluster 99b/let-7e/125a, miR-125a and miR-155. (B) Quantitative RT-PCR data showing the expression levels of the miR-cluster 99b/let-7e/125a members and miR-155 on overexpression relative to the endogenous control snoRNA202. Shown is the mean ± SD. (C) CAFC data showing the number of HSPCs.

these mice, resulting in lethality of some of the recipients (Figure 3D). In 1 of 2 independent transplantation experiments, 6 of 9 miR-cluster 99b/let-7e/125a-overexpressing mice died between 6-30 weeks posttransplantation. In the other transplantation experiment, despite strongly disturbed hematopoietic homeostasis of miR-cluster 99b/let-7e/125a and miR-125a mice, no lethality was observed until 21 weeks posttransplantation.

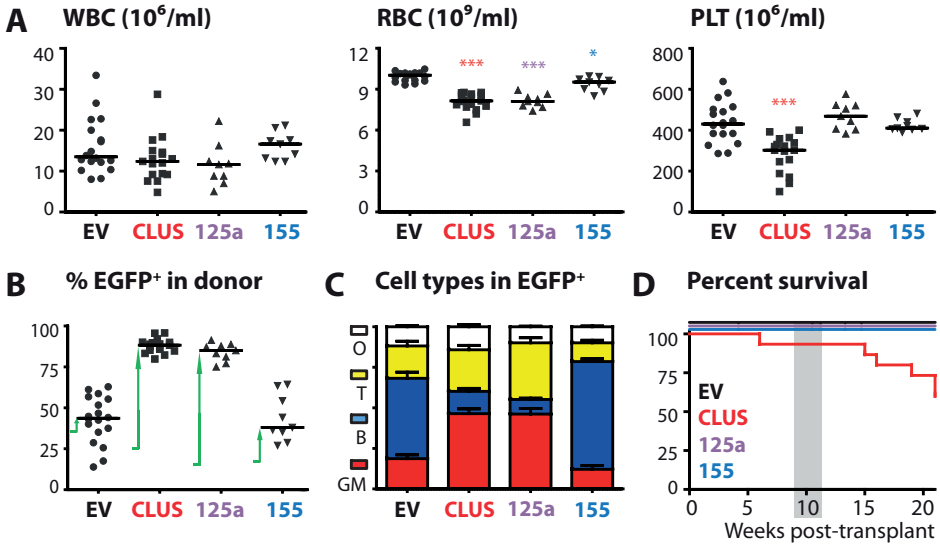

Figure 3. Sustained expression of miR-cluster 99b/let-7e/125a or miR-125a alone disturbs hematopoiesis. (A) WBC, RBC, and platelet counts at 10 weeks after transplantation (n=9-18 mice/group). *P* values (*.01<*P*<.05; ***P* <.001) are displayed (Mann-Whitney test). (B) Chimerism levels were assessed at 10 weeks after transplantation by analyzing the percentage of EGFP⁺ cells in the donor fraction (n=9-18 mice/group). The arrows indicate the increase in chimerism levels over the first 10 weeks after transplantation. (C) Cell-type distribution in the blood as assessed by FACS (n=9-18 mice/group). Shown is the mean ± SEM (D) Survival of mice (n=9-18 mice/group). The gray box indicates the time point analyzed in the other panels. WBC indicates white blood cells; RBC, red blood cells; PLT, platelets; GM, granulocytes/macrophages; B, B lymphocytes; T, T lymphocytes; and O, other cell types.

Competitive advantage provided by miR-cluster 99b/let-7e/125a decreases over time. To quantify the number of HSPCs in the BM compartments of the nonmorbid miR-cluster 99b/let-7e/125a mice and their empty vector controls, CAFC assays on EGFP⁺ BM cells were performed. At 21 weeks posttransplantation, the miR cluster-transduced cells still exhibited more day-35 CAFC activity compared with empty vector-transduced cells (Figure 5A). However, it should be noted that the fold increase in day-35 CAFC activity of miR-cluster 99b/let-7e/125a-overexpressing cells dropped from ~6.500 before transplantation to ~400 in this 21-week time frame *in vivo*. Reduction of the stem cell pool was also suggested by immunophenotypic analyses of the primitive BM compartment. We observed a reduced frequency of EGFP⁺ primitive stem cells in the BM, defined as $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD48^-CD150^+$ cells, in miR-cluster 99b/let-7e/125a mice compared with empty vector mice 5 months after transplantation (Figure 5B-C).

To assess the quality of the HSPCs in the BM compartments of the miR-cluster 99b/let-7e/125a and empty vector mice, we performed secondary BM transplantations. We evaluated the *in vivo* repopulating ability of EGFP⁺ cells isolated from the BM of the primary recipients by transplanting them in competition with freshly isolated BM cells. Whereas miR-cluster 99b/let-7e/125a-overexpressing cells continued to show disturbed hematopoietic homeostasis, reflected by myeloproliferation in the peripheral blood at the expense of the number of B

lymphocytes (Figure 5D), their initial competitive advantage was lost. No differences in EGFP$^+$ donor cell chimerism were observed between miR-cluster 99b/let-7e/125a and empty vector mice 6 weeks or 24 weeks after transplantation (Figure 5E).

To summarize, our data indicate that miR-cluster 99b/let-7e/125a indeed affects HSPC traits. We found that overexpression of this microRNA cluster conferred an apparent initial competitive advantage to engrafting hematopoietic cells in an *in vivo* transplant setting.

In search of functional miR-125a targets. To understand the molecular mechanisms by which miR-cluster 99b/let-7e/125a, and more specifically miR-125a, is able to modulate HSPC fate, it is essential to identify genes regulated by these microRNAs. To address this question, we analyzed Illumina WG6 gene expression arrays from EGFP$^+$ cells transduced with empty vector, miR-cluster, miR-125a and miR-155 (included as a control again). Of the 45.281 probes on the array, 28.977 were expressed in at least 1 of the 4 conditions (empty vector, miR-cluster, miR-125a, and miR-155). For microRNA target prediction, we used one of the most widely used algorithms, namely TargetScan. It predicts 48, 712, and 572 evolutionary conserved targets for miR-99b, let-7e, and miR-125a, respectively.[31] To refine the list, only those targets were selected whose expression reacted on changing microRNA expression levels. In the list of quality-filtered probes (28.977), we identified 912 predicted targets of the entire miR-cluster 99b/let-7e/125a, including 485 specific for miR-125a, and 164 targets of miR-155. We then visualized the expression levels of these predicted targets on overexpression of the miR-cluster 99b/let-7e/125a and miR-155 (Figure 6A). This analysis showed that the expression of most of the predicted miR-cluster 99b/let-7e/125a targets was indeed mildly affected in the miR-cluster-transduced cells, but not in the miR-155–transduced cells. In contrast, most of the miR-155 targets were only affected in the miR-155-transduced cells, but not in the miR-cluster-transduced cells. The entire gene expression dataset is available online (supplemental Table 2) and in GEO (accession number GSE33689).

Because our *in vitro* CAFC activity and *in vivo* transplantation data indicate that miR-125a is responsible for the majority of the miR-cluster 99b/let-7e/125a-induced phenotypes, we restricted our search for downstream targets to only miR-125a. From the list of predicted miR-125a targets (485, identified using Target Scan), we subsequently selected those probes that were at least 1.5-fold differentially expressed and consistently down-regulated upon overexpression of both miR-cluster 99b/let-7e/125a and miR-125a. This resulted in 45 probes representing 40 genes that qualify as candidate functional targets (Table 1). We classified these 40 potential targets using Gene Ontology functional annotations for biological processes. Annotated categories were grouped as illustrated in Figure 6B (supplemental Table 3). Among these groups were cell proliferation, differentiation, and apoptosis. Seven potential miR-125a targets categorized as apoptosis related. Interestingly, among them was *Bak1*, a proapoptotic gene previously suggested by Guo et al as a miR-125a target. Guo et al showed that miR-125a overexpression phenotypes may be mediated via anti-apoptotic effects.[35] This suggests that protection against apoptosis may have been at least partially responsible for the phenotypes that we observed on miR-cluster 99b/let-7e/125a and miR-125a overexpression. However, because enhanced differentiation and lineage skewing were observed in our *in vivo* transplantations, genes involved in cell proliferation and differentiation may be of importance to explain the full spectrum of effects caused by these microRNAs. Future studies are needed to experimentally validate the candidate functional miR-125a targets.

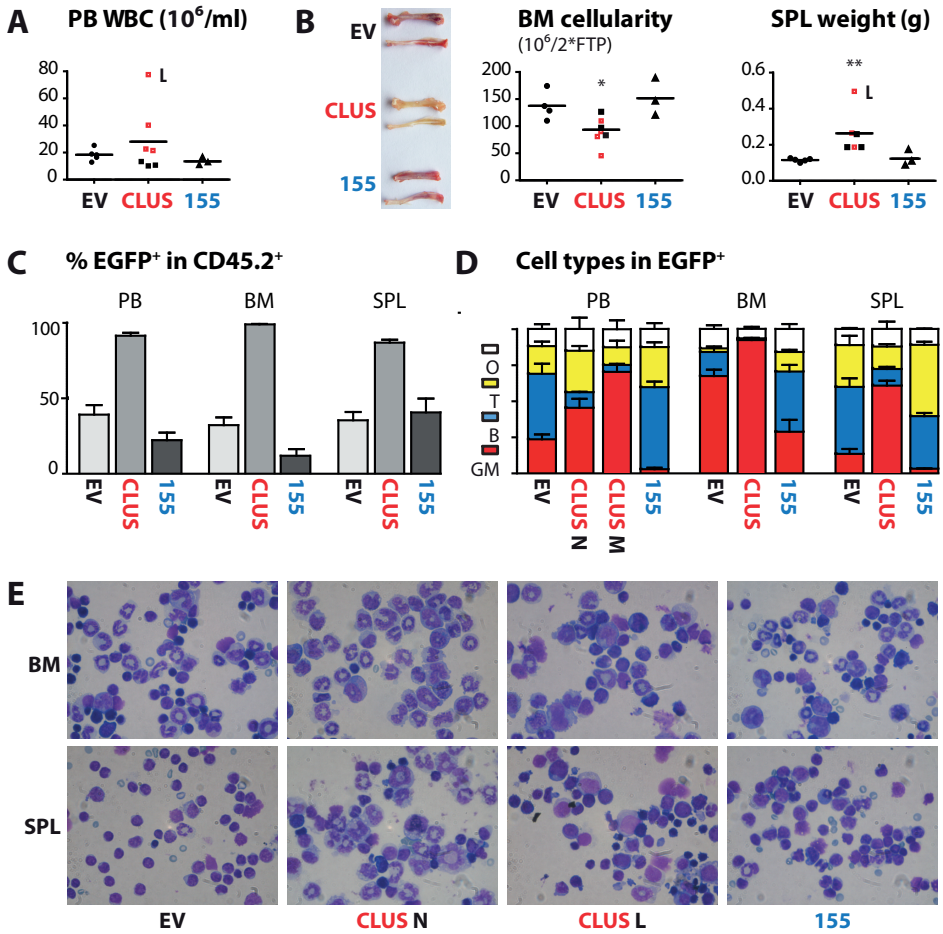

Figure 4. Sustained expression of miR-cluster 99b/let-7e/125a induces MPNs with occasional progression to leukemia. (A) PB WBC counts for empty vector, miR-cluster 99b/let-7e/125a, and miR-155 mice at time of sacrifice. Closed/black squares represent nonmorbid miR-cluster mice, whereas open/red squares represent moribund miR-cluster mice. L indicates leukemic mouse. (B) Photographs of femur and tibia bones, BM cellularity (representing 2 femurs, 2 tibias and pelvic bones) and spleen weight for empty vector, miR-cluster 99b/let-7e/125a and miR-155 mice at time of sacrifice. Closed/black squares represent nonmorbid miR-cluster mice, whereas open/red squares represent moribund miR-cluster mice. *P* values (*.0< *P* <.05; **.001< *P* <.01) are displayed (Mann-Whitney test). L indicates leukemic mouse. (C) Percentage EGFP$^+$ cells in PB, BM, and spleen (n=5 for EV, n=6 for CLUS, n=3 for 155). Nonmorbid and moribund miR-cluster 99b/let-7e/125a mice showed similar percentages and were therefore combined. Shown is the mean ± SEM. (D) Cell-type distribution in PB, BM, and spleen of empty vector, miR-cluster 99b/let-7e/125a, and miR-155 mice as assessed by FACS. Shown is the mean ± SEM. Data for nonmorbid and moribund mice are shown separately for PB (n=5 for EV, n=3 for nonmorbid [N] CLUS, n=3 for moribund [M] CLUS, n=3 for 155) and combined for BM and spleen (n=5 for EV, n=6 CLUS, n=3 for 155), because the cell-type distributions between these mice differed in PB, but not in BM and spleen. (E) Representative MGG-stained cytospin preparations from BM and spleen cells. For the miR-cluster 99b/let-7e/125a, 2 pictures are shown: 1 representative of a nonmorbid mouse (N) and 1 of the leukemic mouse (L).

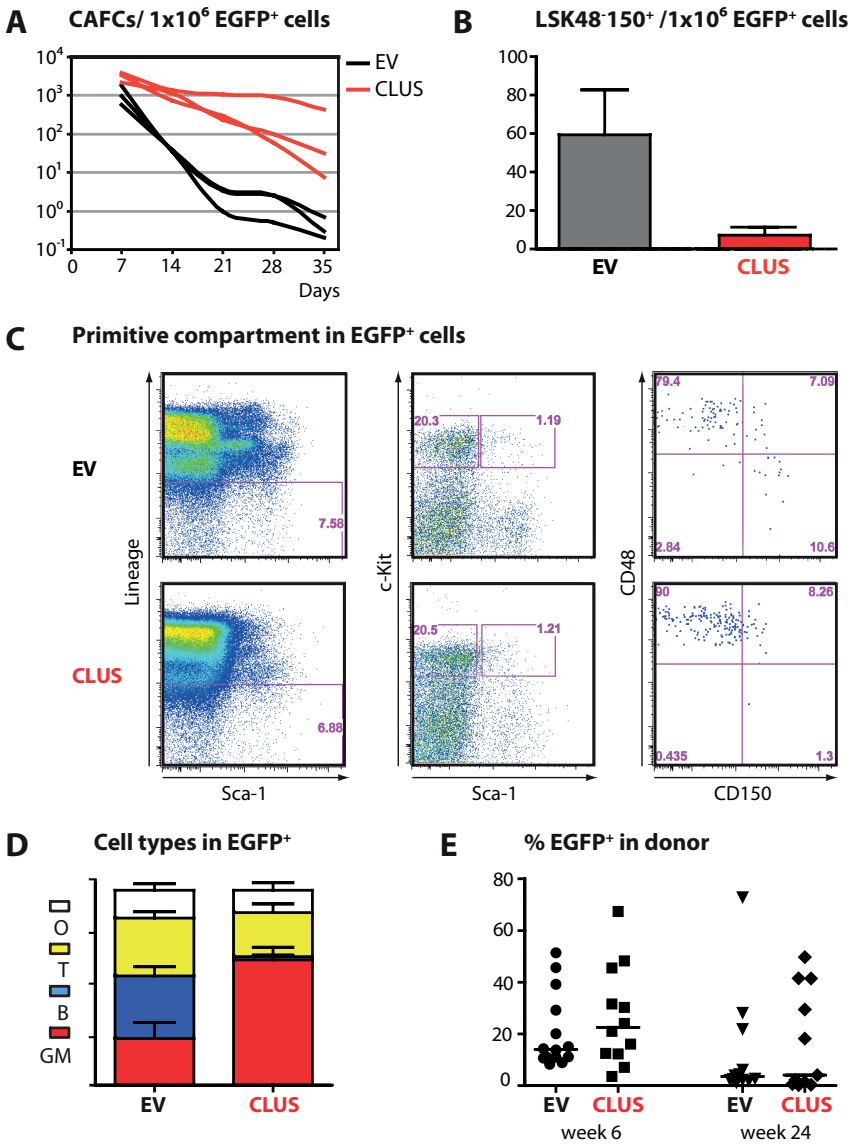

Figure 5. Effects of sustained expression of miR-cluster 99b/let-7e/125a on the stem cell compartment. (A) CAFC data showing the number of HSPCs in the EGFP⁺ BM fraction of 3 individual empty vector and (nonmorbid) miR-cluster 99b/let-7e/125a mice at ~21 weeks posttransplantation. (B) Frequency of LT-HSCs (Lin⁻Sca-1⁺c-Kit⁺CD48⁻CD150⁺) per 1x10⁶ EGFP⁺ BM cells at 21 weeks posttransplantation. Analyses were performed using FlowJo software (TreeStar), followed by quantification of the number of LT-HSCs (n=3/group, same mice as described in panel A). Shown is the mean ± SEM. (C) Representative FACS plots of the EGFP⁺ primitive BM compartment at 21 weeks after primary transplantations. (D) Cell-type distribution in the blood of empty vector and miR-cluster 99b/let-7e/125a mice upon secondary transplantations as assessed by FACS at 24 weeks posttransplantation. Shown is the mean ± SEM (n=15 mice/group). (E) Chimerism levels at 6 and 24 weeks after secondary transplantation (n=15 mice/group). GM indicates granulocytes/macrophages; B, B lymphocytes; T, T lymphocytes; and O, other cell types.

Table 1. Candidate functional miR-125a targets.

Gene Symbol	Fold Change	Gene Ontology category							
		1	2	3	4	5	6	7	8
Mtf1	-5.11				x				
Alpk3	-4.36								x
Mapk8ip1	-3.92	x			x	x		x	
Bcl11b	-3.81	x	x	x	x				
Ube2r2	-3.6						x		
Mfhas1	-3.6								x
Cnnm1	-3.45							x	
Ets1	-3.4	x	x	x	x				
Taf9b	-3.12	x	x		x				
Cramp1l	-3.11								x
Enpep	-3.09		x						
Ache	-3.01			x				x	
Bmpr2	-2.98		x		x	x			
Celsr2	-2.96			x		x			
Itga9	-2.9					x			
Slc8a2	-2.79							x	
St8sia4	-2.72								x
Smcr8	-2.58								x
Ece1	-2.58	x				x			
Tle3	-2.46			x	x	x			
Ubn1	-2.4			x					
Cdr2l	-2.38								x
Atxn1	-2.37				x	x		x	
Baz2a	-2.31				x				
Asah3l	-2.27		x						
Scn5a	-2.26							x	
Lin28b	-2.19				x				
Pafah1b1	-2.02		x	x				x	
Abcc5	-1.91							x	
Bak1	-1.87	x	x	x	x		x	x	
Coro2a	-1.83								x
Cdc42se1	-1.8					x		x	
Foxq1	-1.79	x			x				
Mtus1	-1.78								x
Stard13	-1.77					x			
Nin	-1.76								x
Tspan12	-1.72					x			
Rhobtb2	-1.63					x			
Pctp	-1.62							x	
Ptpn18	-1.55								x

Fold change: down-regulation upon miR-125a overexpression compared to empty vector. Gene Ontology (GO) categories: 1. Apoptosis/Cell death, 2. Proliferation, 3. Differentiation, 4. Chromatin/DNA/RNA, 5. Signal Transduction/Pathways, 6. Protein/Enzymes, 7. Transport, 8.Other (not assigned to category 1-7).

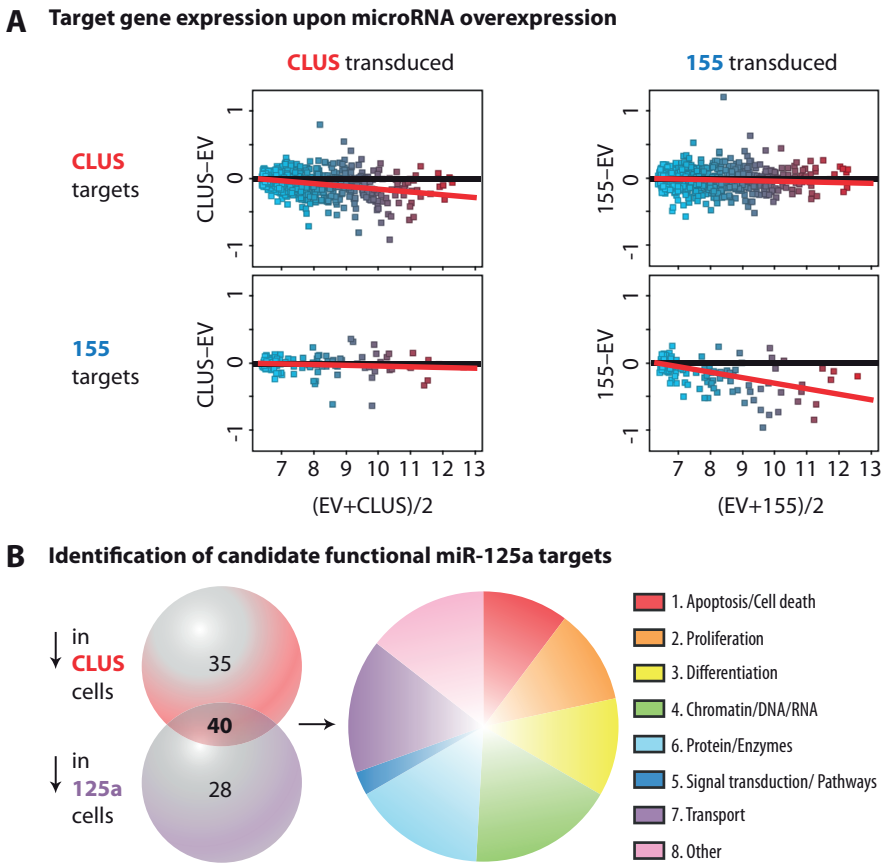

Figure 6. Identification of candidate functional miR-125a targets. (A) Visualization of expression of all quality-filtered predicted miR-cluster and miR-155 targets in miR-cluster-transduced and miR-155-transduced cells. Shown are MvA plots comparing 2 samples. The log2 ratio of each probe (expression difference) is plotted versus the log2 mean for each probe. The red lines represent the best fit to the data. In these plots, the predicted targets that are shared between the miR-cluster and miR-155 are not displayed. (B) Venn diagram showing the identified candidate miR-125a targets down-regulated upon miR-cluster 99b/let-7e/125a or miR-125a overexpression. The intersection represents 40 genes that were consistently 1.5-fold down-regulated upon miR-cluster 99b/let-7e/125a and miR-125a overexpression. These 40 genes were assigned according to Gene Ontology (GO) biological process categories. Genes assigned to common GO categories were grouped together (supplemental Table 3). The pie chart shows relative distributions of grouped GO categories. The size of the sectors in the pie chart was defined based on the number of genes assigned to the category (in redundant fashion). Genes not assigned to any of the defined categories[1-7] were defined as Other.[8] Gene Ontology analyses were performed using GOAL software.[40]

DISCUSSION

In this report, we set out to determine whether the observed natural variation in HSPC traits across the genetically distinct mouse strains B6 and D2 could be due to natural variation in microRNA expression. We performed a genome-wide microRNA expression study of LSK, $LS^{-}K^{+}$,

erythroid and myeloid cells isolated from B6 and D2 mouse strains, and detected natural variation in microRNA expression between these 2 mouse strains. Of special interest was an evolutionary conserved miR-cluster located on chromosome 17 consisting of miR-99b, let-7e, and miR-125a. All cluster members were most highly expressed in LSKs and down-regulated upon differentiation and generally higher expressed in D2 cells compared with B6 cells. We found that overexpression of miR-cluster 99b/let-7e/125a or miR-125a alone conferred an apparent competitive advantage to HSPCs, but also that these cells exhibited increased myeloid differentiation, ultimately leading to the development of MPNs in mice reconstituted with these cells. Loss of competitive advantage following serial transplantation suggests that in the long-term disturbed homeostasis may lead to HSPC exhaustion. Finally, as miR-125a was shown to be responsible for the majority of the miR-cluster 99b/let-7e/125a overexpression phenotypes, we identified candidate functional targets through which miR-125a could modulate HSPC fate.

While we were performing our final experiments, Guo et al reported that miR-99b, let-7e, and miR-125a were enriched in long-term HSCs (LSK/CD34$^-$/Flk2$^-$) and that overexpression of miR-125a alone, but not miR-99b or let-7e, could amplify the HSC pool.[35] This result was shown to be accomplished by decreasing the level of apoptosis in hematopoietic progenitors through targeting pro-apoptotic genes such as *Bak1*. We confirmed the down-regulation of *Bak1* upon miR-cluster 99b/let-7e/125a and miR-125a overexpression, suggesting that decreased apoptosis of HSPCs may have contributed to the observed hematopoietic phenotypes. In line with this finding was the observed prolonged CAFC activity (day 70) and the initial *in vivo* competitive advantage in primary recipients, both observed for the miR-cluster and miR-125a-overexpressing cells. However, whereas Guo et al showed amplification of the HSC pool after sustained miR-125a overexpression, we reported a decline of the primitive BM compartment and loss of competitive advantage upon secondary transplantation. Because anti-apoptotic effects cannot explain these results, we suggest that additional parameters affecting proliferation and differentiation are contributing to the miR-125a-induced phenotypes. As several identified potential miR-125a targets are involved in cell proliferation and differentiation, it would be interesting to determine their role in miR-cluster 99b/let-7e/125a and miR-125a–mediated hematopoiesis.

Other reports have recently described effects of miR-125b, a paralog of miR-125a, on HSPCs. O'Connell et al[36] and Bousquet et al[37] reported that overexpression of miR-125b caused MPNs that progressed to a lethal acute myeloid leukemia or resulted in B/T-cell acute lymphoblastic leukemia. In addition, Bousquet et al provided evidence that miR-125b confers a proliferative advantage to leukemic cells,[37] whereas O'Connell et al showed that miR-125b promotes hematopoietic engraftment of human HSCs.[36] Finally, Ooi et al reported that overexpression of miR-125b in HSCs enhances their function and enriches for lymphoid-balanced and lymphoid-biased HSCs.[38] They also described a skewing toward the lymphoid lineage in the peripheral blood of these mice, and that a small subset of them developed a lymphoproliferative disease. In addition, they identified *Bmf* and *Klf13* as miR-125b downstream targets with a link to apoptosis. Whereas in our dataset we could not confirm down-regulation of *Bmf*, *Klf13* was found to be down-regulated upon overexpression of miR-125a and therefore could be considered as a possible candidate.

It is interesting, yet confusing, to see that all the reports on the miR-125 family in HSCs describe distinct gain-of-function phenotypes (from no- to myeloid- to lymphoid malignancies). As

suggested by O'Connell et al, differences in overexpression levels may underlie these phenotypic differences.[36] In addition, whereas miR-125a and miR-125b have identical seed sequences, the mature forms of these 2 microRNAs differ. Therefore, it needs to be experimentally validated whether miR-125a and miR-125b really do share the exact same targets.

The initial competitive advantage of miR-cluster 99b/let-7e/125a-overexpressing cells and its loss over time relates to the differences between B6 and D2 mice. First, hematopoietic recovery by B6 and D2 stem cells has been studied in competitive transplantation settings and has uncovered interesting kinetics. Whereas D2 hematopoiesis was predominant initially, it was eclipsed by B6 hematopoiesis over time.[39] Second, 2.6% of B6 stem cells has been shown to be in S-phase of the cell cycle versus a striking 24% of D2 stem cells.[3] Together, these findings indicate that the increased proliferation rate for D2 stem cells may have provided them with an initial competitive advantage, but that this increased cycling rate also made them exhaust more rapidly. We consider it plausible that the differential expression of miR-cluster 99b/let-7e/125a (and thus its targets) may at least be in part responsible for the observed variation in stem cell cycling and repopulation kinetics between B6 and D2 mouse strains.

Collectively, our data and other recent reports point to an important role for the miR-125 family in hematopoiesis. To conclude, we uncovered the existence of natural variation in microRNA expression between mouse strains, and showed that this may contribute to the observed natural variation in HSPC traits. Yet, we were not able to identify any published HSPC traits that mapped to the here-reported locus containing miR-cluster 99b/let-7e/125a.[7] We anticipate that future genetic studies will shed new light on how microRNAs modulate HSPC fate and on how they themselves are regulated.

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AUTHORSHIP CONTRIBUTIONS

Contribution: A.G., M.A.W., L.V.B., R.v.O. and G.d.H. designed research; A.G., M.A.W., S.O., E.W. and M.R. performed research; A.G. and M.A.W. analyzed and interpreted data; E.Z. developed Hemdb.org; A.G. and M.A.W. wrote the manuscript with contributions from R.v.O., L.V.B. and G.d.H.

DISCLOSURE OF CONFLICT OF INTEREST

Conflict-of-interest disclosure: The authors declare no competing financial interests. Correspondence: Gerald de Haan, Department of Cell Biology, Section Stem Cell Biology, University Medical Center Groningen, University of Groningen, Groningen 9700 AD, The Netherlands; e-mail: g.de.haan@med.umcg.nl.

REFERENCES

1. De Haan G, Van Zant G. Intrinsic and extrinsic control of hemopoietic stem cell numbers: mapping of a stem cell gene. *J.Exp.Med*. 1997;186(4):529-536.
2. De Haan G, Nijhof W, Van Zant G. Mouse strain-dependent changes in frequency and proliferation of hematopoietic stem cells during aging: correlation between lifespan and cycling activity. *Blood* 1997;89(5):1543-1550.
3. Van Zant G, Eldridge PW, Behringer RR, Dewey MJ. Genetic control of hematopoietic kinetics revealed by analyses of allophenic mice and stem cell suicide. *Cell* 1983;35(3 Pt 2):639-645.
4. Muller-Sieburg CE, Riblet R. Genetic control of the frequency of hematopoietic stem cells in mice: mapping of a candidate locus to chromosome 1. *J.Exp. Med*. 1996;183(3):1141-1150.
5. Morse HC, III, Chused TM, Hartley JW et al. Expression of xenotropic murine leukemia viruses as cell-surface gp70 in genetic crosses between strains DBA/2 and C57BL/6. *J.Exp.Med*. 1979;149(5):1183-1196.
6. Peirce JL, Lu L, Gu J, Silver LM, Williams RW. A new set of BXD recombinant inbred lines from advanced intercross populations in mice. *BMC.Genet*. 2004;57.
7. Gerrits A, Dykstra B, Otten M, Bystrykh L, De Haan G. Combining transcriptional profiling and genetic linkage analysis to uncover gene networks operating in hematopoietic stem cells and their progeny. *Immunogenetics* 2008;60(8):411-422.
8. Bystrykh L, Weersing E, Dontje B et al. Uncovering regulatory pathways that affect hematopoietic stem cell function using 'genetical genomics'. *Nat.Genet*. 2005;37(3):225-232.
9. Gerrits A, Li Y, Tesson BM et al. Expression quantitative trait loci are highly sensitive to cellular differentiation state. *PLoS.Genet*. 2009;5(10):e1000692.
10. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116(2):281-297.
11. Ambros V. The functions of animal microRNAs. *Nature* 2004;431(7006):350-355.
12. Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* 2010;466(7308):835-840.
13. Lim LP, Lau NC, Garrett-Engele P et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 2005;433(7027):769-773.
14. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science* 2004;303(5654):83-86.
15. Xiao C, Calado DP, Galler G et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell* 2007;131(1):146-159.
16. He L, Thomson JM, Hemann MT et al. A microRNA polycistron as a potential human oncogene. *Nature* 2005;435(7043):828-833.
17. Xiao C, Srinivasan L, Calado DP et al. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. *Nat.Immunol*. 2008;9(4):405-414.
18. Zhou B, Wang S, Mayr C, Bartel DP, Lodish HF. miR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. *Proc.Natl.Acad.Sci.U.S.A* 2007;104(17):7080-7085.
19. Johnnidis JB, Harris MH, Wheeler RT et al. Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature* 2008;451(7182):1125-1129.
20. Fazi F, Rosa A, Fatica A et al. A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell* 2005;123(5):819-831.
21. O'Connell RM, Rao DS, Chaudhuri AA et al. Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J.Exp.Med*. 2008;205(3):585-594.
22. Thai TH, Calado DP, Casola S et al. Regulation of the germinal center response by microRNA-155. *Science* 2007;316(5824):604-608.
23. Rodriguez A, Vigorito E, Clare S et al. Requirement of bic/microRNA-155 for normal immune function. *Science* 2007;316(5824):608-611.
24. Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. *Nat.Rev.Cancer* 2006;6(4):259-269.
25. Kluiver J, Kroesen BJ, Poppema S, Van den Berg A. The role of microRNAs in normal hematopoiesis and hematopoietic malignancies. *Leukemia* 2006;20(11):1931-1936.
26. Sturn A, Quackenbush J, Trajanoski Z. Genesis: cluster analysis of microarray data. *Bioinformatics*. 2002;18(1):207-208.
27. Mao TK, Chen CZ. Dissecting microRNA-mediated gene regulation and function in T-cell development. *Methods Enzymol*. 2007;427171-189.
28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25(4):402-408.
29. Van Os RP, Dethmers-Ausema B, De Haan G. In vitro assays for cobblestone area-forming cells, LTC-IC, and CFU-C. *Methods Mol.Biol*. 2008;430143-157.

30. Ploemacher RE, Van der Sluijs JP, Van Beurden CA, Baert MR, Chan PL. Use of limiting-dilution type long-term marrow cultures in frequency analysis of marrow-repopulating and spleen colony-forming hematopoietic stem cells in the mouse. *Blood* 1991;78(10):2527-2533.
31. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005;120(1):15-20.
32. Popovic R, Riesbeck LE, Velu CS et al. Regulation of mir-196b by MLL and its overexpression by MLL fusions contributes to immortalization. *Blood* 2009;113(14):3314-3322.
33. Zhan M, Miller CP, Papayannopoulou T, Stamatoyannopoulos G, Song CZ. MicroRNA expression dynamics during murine and human erythroid differentiation. *Exp.Hematol.* 2007;35(7):1015-1025.
34. Baskerville S, Bartel DP. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. *RNA*. 2005;11(3):241-247.
35. Guo S, Lu J, Schlanger R et al. MicroRNA miR-125a controls hematopoietic stem cell number. *Proc.Natl.Acad.Sci.U.S.A* 2010;107(32):14229-14234.
36. O'Connell RM, Chaudhuri AA, Rao DS et al. MicroRNAs enriched in hematopoietic stem cells differentially regulate long-term hematopoietic output. *Proc.Natl.Acad.Sci.U.S.A* 2010;107(32):14235-14240.
37. Bousquet M, Harris MH, Zhou B, Lodish HF. MicroRNA miR-125b causes leukemia. *Proc.Natl.Acad.Sci.U.S.A* 2010
38. Ooi AG, Sahoo D, Adorno M et al. MicroRNA-125b expands hematopoietic stem cells and enriches for the lymphoid-balanced and lymphoid-biased subsets. *Proc.Natl.Acad.Sci.U.S.A* 2010
39. Van Zant G, Holland BP, Eldridge PW, Chen JJ. Genotype-restricted growth and aging patterns in hematopoietic stem cell populations of allophenic mice. *J.Exp.Med.* 1990;171(5):1547-1565.
40. Volinia S, Evangelisti R, Francioso F, Arcelli D, Carella M, Gasparini P. GOAL: automated Gene Ontology analysis of expression profiles. *Nucleic Acids Res*. 2004;32:W492-W499.

Supplemental information available online on request

Table S1. MicroRNA expression dataset on four cell types isolated from B6 and D2 mice.

Table S2. Gene expression dataset on microRNA-overexpressing BM cells.

Table S3. Gene Ontology classification of miR-125a targets indentified in microRNA-overexpressing cells.

CHAPTER 4

THE COMBINATION OF VALPROIC ACID AND LITHIUM PREVENT HEMATOPOIETIC STEM/PROGENITOR CELL DIFFERENTIATION

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ABSTRACT

Despite increasing knowledge on the regulation of hematopoietic stem/progenitor cell (HSPC) self-renewal and differentiation, *in vitro* control of stem cell fate decisions has been difficult. The ability to inhibit HSPC commitment in culture may be of benefit to cell therapy protocols. Small molecules can serve as tools to manipulate cell fate decisions. Here, we tested two small molecules, valproic acid (VPA) and lithium (Li), to inhibit differentiation. HSPCs exposed to VPA and Li during differentiation-inducing culture preserved an immature cell phenotype, provided radioprotection to lethally irradiated recipients, and enhanced *in vivo* repopulating potential. Anti-differentiation effects of VPA and Li were observed also at the level of committed progenitors, where VPA re-activated replating activity of common myeloid progenitors and granulocyte macrophage progenitors. Furthermore, VPA and Li synergistically preserved expression of stem cell-related genes and repressed genes involved in differentiation. Target genes were collectively co-regulated during normal hematopoietic differentiation. In addition, transcription factor networks were identified as possible primary regulators. Our results show that the combination of VPA and Li potently delays differentiation at the biologic and molecular levels and provide evidence to suggest that combinatorial screening of chemical compounds may uncover possible additive/synergistic effects to modulate stem cell fate decisions.

INTRODUCTION

Hematopoietic stem/progenitor cells (HSPCs) have a great clinical potential, but many of their applications are limited by insufficient numbers. Therefore, attempts have been made to define culture conditions, which would amplify stem cells in an uncommitted state and maintain their potential *in vivo*.[1-3] However, so far there has been limited success in preventing HSPC commitment and differentiation *in vitro* without genetic manipulation (eg, overexpression of *HoxB4*, *Bmi1*, β-*catenin*).[4-6] Alternatively, small molecules could be used as new tools for manipulating cell fate decisions. A number of these compounds have been identified to affect developmental processes, stem cell self-renewal and differentiation, or reprogramming efficiency of somatic cells to a pluripotent state.[3,7-11] Two of such molecules, valproic acid (VPA) and lithium (Li), have been shown to affect normal and malignant hematopoiesis.[12,13] VPA is a pleiotropic histone deacetylase (HDAC) inhibitor (HDI), which can modulate histone acetylation by preventing its deacetylation.[8] During commitment and differentiation, the chromatin structure of stem cells undergoes major epigenetic changes, which affect cellular transcriptional programs. Therefore, factors interfering with chromatin remodeling enzymes, such as HDIs, might modulate the behavior of stem and progenitor cells.[14,15] For instance, it has been suggested that VPA can enhance stem cell proliferation, while retaining self-renewal in *ex vivo* stem cell expansion protocols.[16-18] Furthermore, several groups suggested that HDIs can affect multilineage differentiation of hematopoietic cells.[19-22] The hematopoietic effects of Li have been described for the first time in psychiatric patients, which included disturbed differentiation into several lineages and increased numbers of $CD34^+$ cells.[13,23,24] *In vivo* and *in vitro* studies with human and murine BM cells confirmed the stimulatory effects of Li on hematopoiesis.[25,26] Furthermore, experimental findings suggest that Li can increase transplantable HSPCs in mice.[25] Although the mechanism by which Li affects HSPCs is unclear, multiple molecular targets have been proposed, including glycogen synthase kinase 3β, a negative regulator of Wnt pathway.[27] Wnt signaling, as well as epigenetic events, play an important role in control of HSPC fate decisions.[6,14] Taken together, data suggest that VPA and Li might serve as chemical regulators of both stem cell maintenance and differentiation.

We hypothesized that combinations of small molecules might be useful to promote maintenance of HSPCs in culture by inhibiting cell commitment. In this study, the combined effects of VPA and Li on HSPC proliferation, differentiation, and self-renewal were investigated. We document potent synergistic effects of both chemical compounds in a variety of *in vitro* and *in vivo* assays and showed strong anti-differentiation activity at the level of uncommitted and committed progenitors. Moreover, we identified genetic networks and putative targets that are transcriptionally affected by both compounds. Our data support the notion that chemical compounds may be of use in *ex vivo* cell therapy protocols.

MATERIAL AND METHODS

Mice. Female B6.SJL (CD45.1) mice, originally purchased from The Jackson Laboratory and bred in our local animal facility, and female wild-type B6 (CD45.2) mice (Harlan) were used as a source of donor HSPCs. In the transplantation settings, we used female B6.SJL donors, female wild-type

B6 (Harlan) BM as competitors, and female F1:B6xB6.SJL (CD45.1/2) as recipients. All animal experiments were approved by the local animal ethical committee of the University of Groningen.

Stem/progenitor cell isolation. Mice were anesthetized and killed by cervical dislocation. Unfractionated BM cells were obtained by crushing femora, tibia, and pelvic bones. Cell suspensions were filtered through a 100µm cell strainer (BD Biosciences) to remove debris, and the cells were counted on a Medonic CA620 analyzer (A. Menarini Diagnostics). After erythrocyte lysis cells were stained with lineage cocktail (A700-Mac1, A700-Gr1, A700-Ter119, A700-CD3, A700- B220), Pacific Blue-Sca1, PE-ckit, FITC-CD34, and PECy7-CD16/32 (Biolegend). After staining, the cells were resuspended in propidium iodide (PI) solution (1µg/mL) and uncommitted ($Lin^-Sca1^+ckit^+$ [LSK]) and committed (common myeloid progenitor [CMP], $L^-S^-K^+CD34^+CD16/32^{mid}$; granulocyte macrophage progenitor [GMP], $L^-S^-K^+CD34^+CD16/32^{high}$) hematopoietic stem/progenitor subpopulations were sorted by aMoFlo XDP cell sorter (Dako Cytomation).

Stem/progenitor cell differentiation. Freshly isolated uncommitted and committed hematopoietic progenitor subpopulations were differentiated in a liquid or semisolid medium in the presence of FCS (10% or 30% FCS, respectively), murine SCF (mSCF; 100 ng/mL; Amgen), and recombinant mGM-CSF (10 ng/mL or 20 ng/mL, respectively; R&D Systems). Cells in liquid cultures were plated at a density of 2000 cells/mL in a 6-well plate (Costar) and cultured in StemSpan medium (StemCell Technologies). Cells in semisolid medium were plated in various concentrations. The cells were cultured for 7 days at 37°C in a humidified atmosphere and 5% CO_2 in air with or without 1mM VPA (Sigma-Aldrich) or 5mM LiCl (J.T.Baker), or the combination. Both compounds were dissolved in PBS solution (PAA Laboratories GmbH).

Cell analysis. After liquid cultures, cells were harvested; total cell number and cell viability were determined by trypan blue and/or PI exclusion. Cultured cells were centrifuged for cytospin preparations (800 rpm; Shandon cytospin III cytocentrifuge; Thermo Fisher Scientific BV,) and stained with May-Grunwald-Giemsa staining. Cytospins were analyzed with light microscopy by counting >200 cells per experimental condition. Cells were classified as immature (blast-like) or differentiated on the basis of nucleus and cytoplasm morphology. To analyze the immunophenotype of 7-day differentiated LSK cells, cells were stained with a cocktail of Abs as described earlier and analyzed on the LSR-II (BD Bioscience). Data analyses were performed with FlowJo PC Version 7.6.5 software (TreeStar).

Clonogenic assays. The CFU assay was performed to assess the potential of cells to form granulocyte/macrophage colonies (CFU-GM). Briefly, either freshly isolated hematopoietic progenitor subpopulations or cells differentiated for 7 days were plated in a methylcellulose medium, and after 6 or 7 days colonies with granuloid and/or macrophage cells were scored. Colonies derived from single cells of freshly isolated progenitors were replated, and secondary GM colonies were scored after another 7 days of semisolid culture. To assess the number of hematopoietic progenitor cells or more primitive stem cells cobblestone area-forming cell (CAFC) assays were performed in the population of 7-day differentiated cells. Both assays were performed as described previously.[28]

***In vivo* transplantation assays.** Seven-day cultured cells (CD45.1) alone or mixed with $0.5x10^6$ of freshly isolated BM competitor cells (CD45.2) were injected into the retro-orbital sinus of lethally irradiated (9.5 Gy) mice (CD45.1/2). In transplantations without competitors, recovery of peripheral blood counts was determined up to 32 days by automatic cell counting of blood samples (Medonic CA620 analyzer). Long-term repopulation potential of cells transplanted in the presence of competitors was monitored by calculation of the percentage of CD45.1, CD45.2, and CD45.1/2-derived cells in the blood. Data were acquired with an LSR-II (BD Bioscience) and analyzed with FlowJo PC Version 7.6.5 software (TreeStar).

Gene expression analysis. Global gene expression was assessed in 7-day cultured cells (growth factor [GF] only, Li, VPA, Li+VPA). All samples were analyzed in independent biologic triplicates. Total RNA was isolated with the RNeasy kit (QIAGEN), according to manufacturer's protocol. RNA concentration, quality, and integrity were measured with the Experion Automated Electophoresis System (Bio-Rad). RNA was amplified with the Illumina TotalPrep RNA Amplification Kit (Ambion/Applied Bioscience) and hybridized to Mouse Ref-8_V2 expression platform (Illumina) according to the manufacturer's instruction. Scanning was performed on the iScan System (Illumina). Image analyses and extraction of raw expression data were performed with BeadStudio Version 3.2 software (Illumina) with default settings, no background subtraction, and no normalization. The raw data were threshold at 1, log2-transformed and quantile-normalized with the use of GeneSpring-GX11.0 (Agilent). From the starting probe list (25 697 probes) probes not expressed in any replicate of the 4 conditions were excluded. The filtered list represented 20586 probes. Probes differentially expressed between drug-treated and control sample (GFs only) were defined by Welch *t* test with Benjamini-Hochberg correction and false discovery rate <0.05. Next, a 1.5-fold differential expression cutoff was applied. Gene expression analyses of 4 distinct primary cell subsets (LSK, $L^-S^-K^+$, $Gr1^+$, $Ter119^+$), as described previously,[29] were used for comparison of VPA and Li effects with the physiologic hematopoietic differentiation program. Finally, data were subjected to relevance network analyses as described in Voy at al.[30] Networks were visualized in Gephi Version 0.8 beta software with the use of the Yifan-Hu and Fruchterman-Reingold force-directed layout algorithm.[31] All raw data were deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) as accession number GSE34088.

ChIP assay. The ChIP assay was performed to determine histone acetylation at promoter regions of specific genes with the use of the method described by Frank et al.[32] In brief, $5x10^6$ cells were fixed with 1% formaldehyde for 10 minutes at RT. After cell lysis, cross-linked chromatin was fragmented by sonication to obtain ~200- to 600-bp fragments. Precleared chromatin was precipitated with anti–acetyl-Histone4 (Millipore) Ab. The immunoprecipitated chromatin was washed subsequently with washing buffers.[32] For reverse cross-linking, samples were incubated overnight at 65°C in 1% SDS, 0.1M NaHCO-containing buffer, followed by treatment with Rnase A (Roche) and proteinase K (Fermentas Gmbh). DNA was isolated by ethanol/chloroform/isopropanol precipitation. The promoter regions of genes of interest were amplified by quantitative PCR (Bio-Rad) with the use of gene-specific primer pairs for β-actin (fwd, GGCAGTGTCCACAAGGGCGG; rev, TTGAGGAAGAGGATGAAGAGTTTTGGCG), HoxA7

(fwd, GCCAGTCTTCCAGCATGGCCTG; rev, CTCTGCTGCCCAACGCTCTCTG), and HoxB4 (fwd, GGGAGGGGTAGAGAAGGGGAAATAAACC; rev, GCCACCCGGCCTGCGATTC).

RESULTS

VPA and Li preserve the immature phenotype of HSPCs during myeloid differentiation *in vitro*. *In vitro* differentiation cultures are a convenient model to study the effects of new compounds on cell proliferation and differentiation. Purified HSPCs, defined by a LSK phenotype, were differentiated into the granulocyte/macrophage lineage by stimulation with SCF and GM-CSF in the presence or absence of VPA with or without Li. After a 7-day liquid cultures the effect of both compounds on cytokine-induced HSPC differentiation was studied with respect to cell proliferation and differentiation.

The addition of Li, either alone or in combination with VPA, led to lower numbers of nucleated cells, whereas this effect was not observed on VPA exposure (Figure 1A). Despite decreased proliferation, the combination of VPA+Li resulted in a significantly higher proportion of viable cells as measured by PI exclusion, used as a marker for dead cells (Figure 1B). Because the cultures were initiated with purified LSK cells, after 7 days of differentiation Lineage, Sca-1, and c-kit marker expression was reanalyzed by flow cytometry. Combined drug treatment (VPA+Li) resulted in a significantly higher (8-fold) number of cells with a LSK phenotype compared with control cells grown with GFs only. On VPA exposure a 6-fold increase in number of phenotypically defined LSK cells was observed, whereas Li had only a marginal (1.5-fold) effect (Figure 1C-D). The immature cell phenotype as measured by flow cytometry was in the agreement with morphology of the cells (Figure 1E-F). Quantification of the percentage of cells with blast-like morphology showed that cells treated with VPA+Li contained 7.5-fold more cells with immature morphology. VPA treatment lead to a 4.5-fold increase, whereas the addition of Li resulted in only 2.5-fold more cells with blast-like morphology over control conditions (Figure 1E-F). These data indicate that VPA and Li treatment preserved the HSPC phenotype and morphology after strong differentiation-inducing cultures.

Enhanced *in vitro* functional potential of cells exposed to VPA and Li. To test whether cells exposed to VPA and Li retained stem/progenitor function, *in vitro* assays for hematopoietic cell activity were performed. Progenitor potential of 7-day cultured cells was determined with semisolid CFU assay, whereby cells were stimulated into granulocyte/macrophage colonies with GM-CSF and SCF. Cells treated with VPA alone or with a combination of VPA+Li gave rise to a higher number of colonies than control cells (45-fold and 81-fold more, respectively). However, treatment with Li did not significantly influence colony-forming capacity (Figure 2A). To test the functional activity of more primitive cells after 7 days of differentiation culture, we performed CAFC assays. In this assay, progenitor activity is measured by early appearing CAFCs (day-7) and stem cell activity by late appearing CAFCs (day 35). Cells exposed to the combination of both compounds (VPA+Li) showed a 30-fold increase in absolute numbers of early- and a 3.5-fold increase in absolute numbers of late-appearing CAFCs (P<.05) compared with control cells (GFs only). Cells treated with VPA alone showed 7-fold and 2-fold more early and late CAFC activity (P<.05), respectively. In contrast, for Li-treated cells no enhancement of early or late

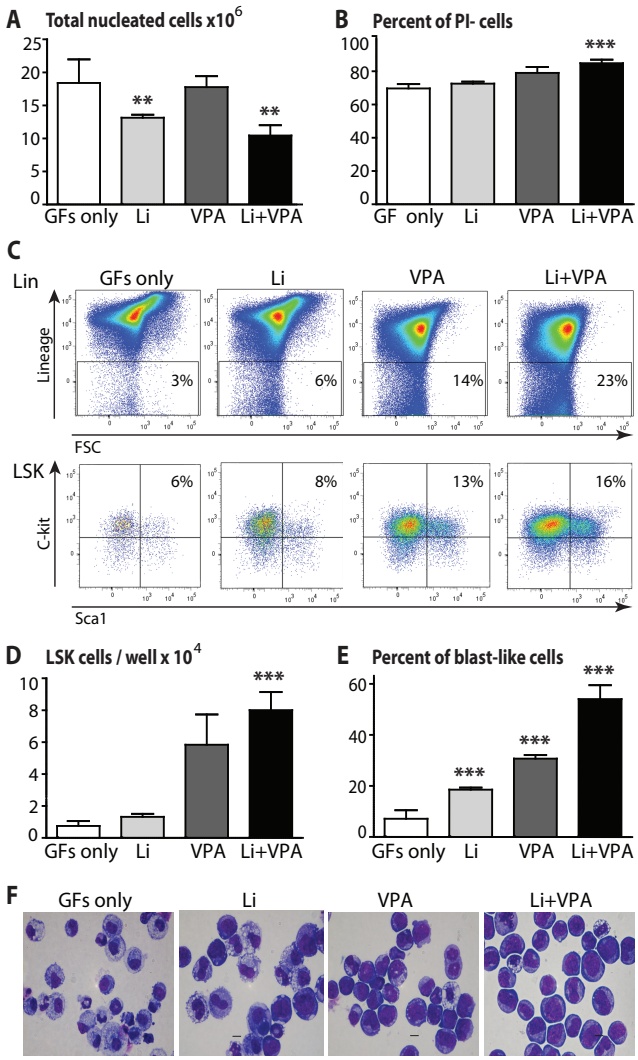

Figure 1. Preserved immature phenotype of hematopoietic progenitor cells after *in vitro* myeloid differentiation culture. HSPCs (LSK cells) were differentiated *in vitro* into the myeloid lineage for 7 days in the presence or absence of VPA and/or Li. (A) Effects of VPA+Li treatment on cell proliferation. The number of viable cells was scored with trypan blue exclusion. Shown is the mean ± SD (n = 5-7). (B) The proportion of cells that excludes PI after 7 days of differentiation culture. Shown is the mean ± SD (n = 5-7). The proportion of cells excluding PI was in agreement with the proportion of dead cells stained with trypan blue (data not shown). (C) Representative flow cytometric analysis of LSK expression on cells cultured for 7 days with or without VPA+Li. Cells in the graphs were pregated for viable (PI-) cells. An equal number of events were collected for each sample. (D) Absolute number of cells with preserved LSK phenotype, quantified on the basis of on FACS data, presented as number of LSK cell per well. Error bars represent SD of the mean (n = 3-7). (E) Percentage of cells with preserved blast-like morphology after 7-day differentiation culture. Shown is the mean ± SD (n = 3-6). (F) Representative cell morphology of cells cultured with or without VPA+Li. Cytospin preparations were stained with May-Grunwald-Giemsa and viewed under a light microscope (original magnification X100). The differences between groups were evaluated by Welch *t* test, $^{**}P < .01$ and $^{***}P < .001$.

CAFC activity was observed (Figure 2B). As shown by these 2 independent assays, the *in vitro* functional potential of stem and progenitor cells was significantly enhanced by VPA and VPA+Li treatment, but the combination of both compounds most efficiently preserved HSPC function.

VPA and Li improve survival, blood cell recovery, and short-time engraftment after BM transplantation. To confirm the functional activity observed with *in vitro* assays, the *in vivo* reconstitution properties of cells differentiated in the presence of VPA and/or Li were examined. The progeny of 2000 LSK cells, cultured for 7 days with GM-CSF with or without VPA and Li, was transplanted into lethally irradiated mice. At days 10, 14, 21, 28, and 32 after transplantation survival and recovery of peripheral blood counts were analyzed. Mice that received a transplant with cells exposed to VPA+Li had significantly (P<.05) improved survival (12 of 14 mice survived) compared with control mice that received cells cultured with GFs only (6 of 14 mice survived; Figure 3A). In addition, blood cell counts showed that the red blood cell numbers and platelet numbers recovered faster in recipients of VPA+Li-treated cells compared with control mice, confirming more progenitor cell activity in the transplanted cell population (Figure 3B-C). In contrast, mice reconstituted with cells cultured in the presence of a single drug, either VPA or Li, did not show improved survival or blood recovery over mice that received a transplant with cells differentiated with GFs only (supplemental Figure 1). These data strongly indicate that the combination of VPA+Li most potently preserved immature, radioprotective hematopoietic progenitor cells.

To evaluate the effects of VPA+Li treatment on long-term repopulating capacity, the cultured progeny of 2000 LSK cells was transplanted into lethally irradiated recipients together with freshly isolated BM competitor cells. Recipient mice transplanted with cells cultured in the presence of VPA+Li displayed higher chimerism levels through the entire time course of the experiment compared with mice that received a transplant with cells cultured in the presence of GFs only. However, the differences between the 2 groups decreased over time (Figure 3D). The percentage of chimerism was significantly higher in the VPA+Li group up to 13 weeks after

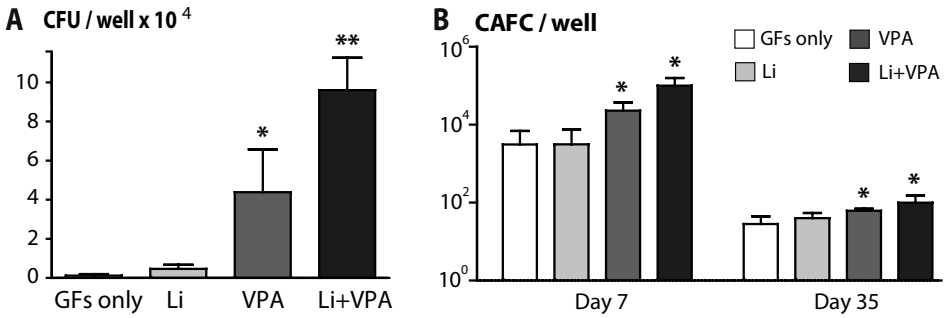

Figure 2. Enhanced *in vitro* function of cells differentiated in the presence of Li and VPA. Cells differentiated *in vitro* with or without VPA and/or Li were assayed for progenitor/stem cell activity. (A) CFU-GM data showing effect of the compound treatment on the total number of colonies. Shown is the mean ± SD. (B) CAFC data showing progenitor (day-7) and stem cells (day-35) activity of 7-day cultured cells. The differences between groups were evaluated by Welch *t* test, **P* <.05 and ***P* <.01.

transplantation, suggesting robust preservation of short-term repopulating stem/progenitor cells. Furthermore, 28 weeks after transplantation only 2 of 5 recipients transplanted with cells cultured with GFs only reached chimerism levels > 1%, whereas > 1% of donor cell contribution was observed for all mice (5 of 5) in the VPA+Li group. Chimerism levels remained stable and distinguishable between the 2 groups until 36 weeks (supplemental Figure 1). In addition, treatment with VPA+Li did not influence the multilineage capacity of the cells because all mature blood types were found in comparable ratios (supplemental Figure 1). Collectively, these data strongly indicate that addition of VPA+Li to differentiation-inducting cultures preserved cells with *in vivo* repopulation potential.

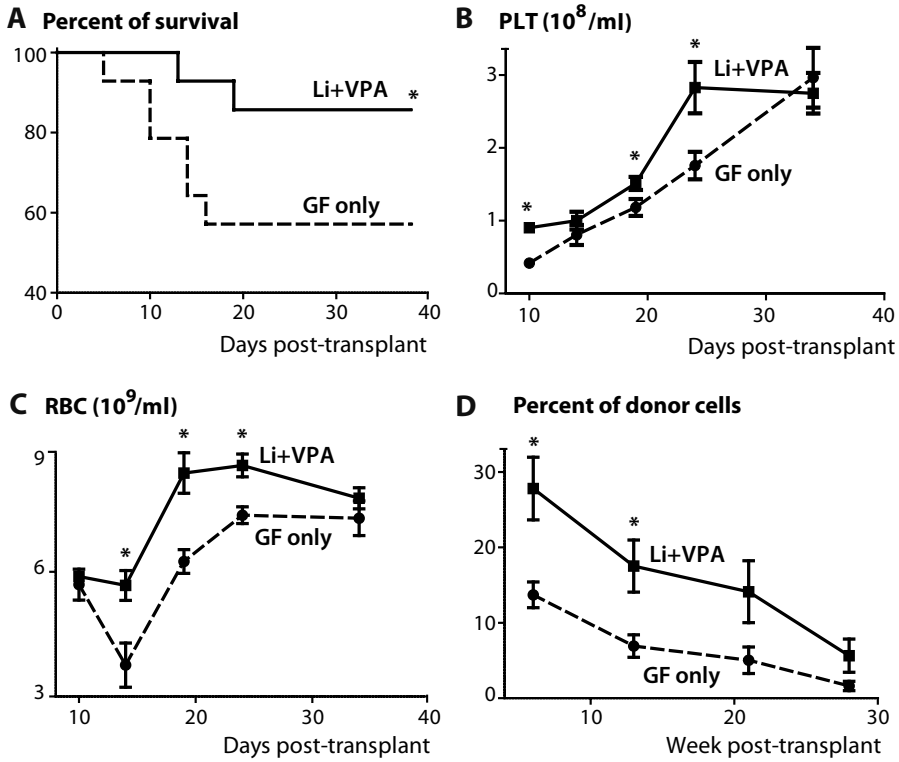

Figure 3. Improved radioprotection and short-time engraftment on BM transplantation with cells exposed to VPA+Li. LSK cells cultured for 7 days with GM-CSF and SCF in the presence or absence of VPA+Li were transplanted into lethally irradiated recipients with or without competitors. (A) Survival of the recipients was screened for 40 days after noncompetitive transplantations. Significance was calculated with Mantel-Cox test (P = .043) and Gehan-Breslow-Wilcoxon test (P = .0394). (B) Platelet counts in the peripheral blood of recipient mice at 10 to up to 40 days after transplantation. Error bars represent SEM. (C) Red blood cell counts in the peripheral blood of recipient mice at 10 to up to 40 days after transplantation. Error bars represent SEM. (D) LSK cells cultured for 7 days were competitively transplanted with fresh BM cells into lethally irradiated recipients. Percentage of donor cells in the peripheral blood of recipient mice up to 28 weeks after transplantation. Error bars represent SEM (n = 5). The differences between groups were evaluated by Welch t test, $^*P$ <.05.

VPA increases proliferation and self-renewal of committed myeloid progenitors. Since our results indicate that VPA and Li enhanced short-term repopulating progenitor cells, the effects of the compound treatment on distinct progenitor cell subsets were characterized. To address this question we isolated by flow cytometry uncommitted (LSK) and committed myeloid (CMP and GMP) cell subsets and performed methylcellulose assays for each sorted cell population (Figure 4A). Cells were stimulated to differentiate with SCF and GM-CSF in the presence of VPA and/or Li. VPA and VPA+Li decreased colony-forming activity of LSK cells, showed no effect on clonogenic activity of CMP cells, but displayed strong potentiating effect on colony-forming ability of the GMP cell population (Figure 4A). GMPs had a limited colony-forming ability (~40 colonies per 1000 cells), which increased in the presence of VPA and VPA+Li by almost 8-fold. Li, as a single agent, showed no effect on colony-forming ability of the tested progenitor cell populations.

To investigate whether the observed effects on clonogenic activity were caused by either proliferation or cell death (or both), single-cell proliferation assay was performed. Single cells of each distinct progenitor population (LSK, CMP, GMP) were sorted into 96-well plates in liquid differentiation medium containing SCF and GM-CSF and were cultured in the presence or absence of VPA and/or Li for 7 days. Cell death was not significantly affected by drug treatment in any of the tested cell populations (Welch *t* test, $P > .05$; Figure 4B). Cell proliferation was quantified by scoring the size of the clone grown from a single cell. Clone size was classified as small, medium, or large. Less than 40% of GMPs at the single-cell level were able to grow into medium or large colonies, whereas on addition of VPA and VPA+Li > 80% of colonies grow into larger (medium + large) colonies. The enhancing effect of VPA and VPA+Li on cell proliferation was also apparent at the level of CMPs, however, to a lesser extent. Surprisingly, VPA and VPA+Li decreased proliferative capacity of the LSK cells. At the single-cell level Li alone showed no effects on the proliferation of distinct hematopoietic cell populations (Figure 4B). These data indicate that VPA displayed a major effect on cell proliferation, which was potentiated in the presence of Li.

Next, the effect of VPA treatment on plating efficiency of distinct cell progenitor populations was tested with the use of single-cell CFU assay. Single cells of each of the 3 progenitor populations were sorted into 96-well plate in semisolid medium with SCF and GM-CSF and cultured with or without VPA and/or Li. VPA and VPA+Li decreased the plating efficiency of LSK cells, did not influence CMP plating efficiency, but significantly increased the plating efficiency of GMP at the single-cell level (in accordance with Figure 4A). Strikingly, whereas only 10% of purified GMPs were able to produce colonies in the presence of GFs only, the addition of VPA or VPA+Li increased clonogenic activity of GMPs to 60% (Figure 4C). Next, the self-renewal potential of compound-exposed cells was determined by replating colonies derived from a single cell. From each group, approximately 30 individual colonies were harvested and replated to new methylcellulose cultures in the presence or absence of VPA and/or Li. In the presence of GF only ~50% of individual colonies formed by single LSK cells were able to generate secondary colonies, whereas on exposure to VPA and VPA+Li 100% of the replated colonies derived from single LSK cells gave rise to secondary colonies. Furthermore, VPA and VPA+Li not only enhanced LSK replating efficiency of individual colonies but also increased the total number of secondary colonies derived from a single cell. Strikingly, although CMP and GMP cells did not show any replating activity by themselves, the addition of VPA and VPA+Li induced replating

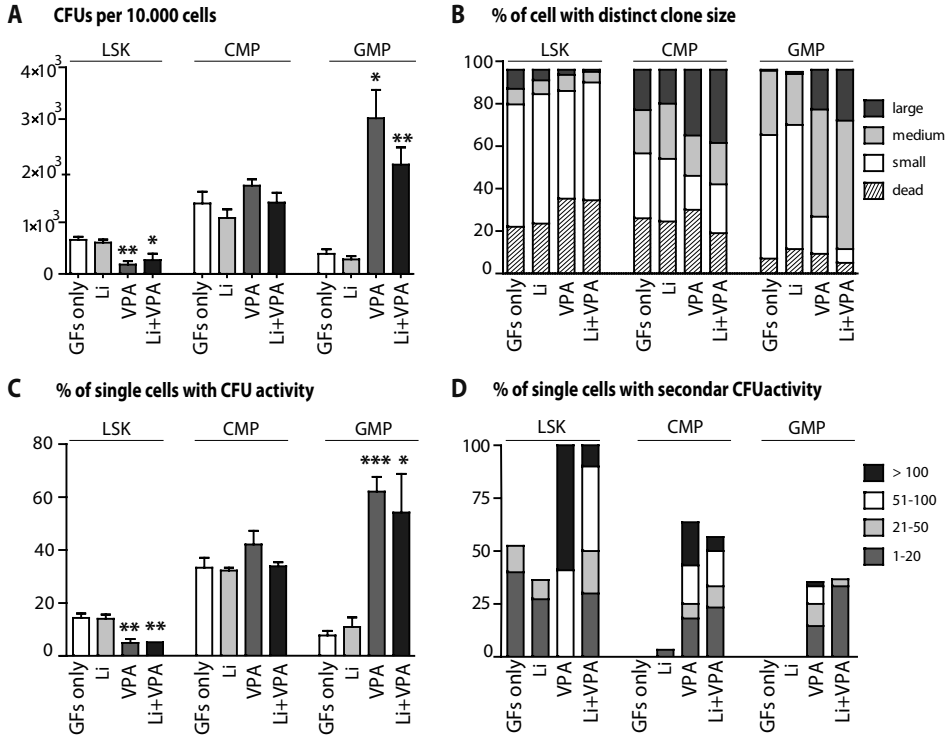

Figure 4. Increased proliferation and induction of replating activity of committed myeloid progenitors on VPA and VPA+Li treatment. Primary myeloid progenitor subpopulations were sorted by flow cytometry, and the effect of VPA+Li exposure on proliferation, colony-forming ability, and self-renewal potential was tested. (A) Influence of VPA and/or Li on CFU-GM activity of distinct myeloid subpopulations. Data are presented as colony forming cells per 10 000 cells. Shown is the mean ± SD. (B) Single-cell proliferation potential of distinct myeloid progenitor subpopulations in liquid culture with SCF and GM-CSF in the presence or absence of VPA and Li. After 7 days of culture, the number of wells with dead cells and the size of clones derived from single cells were scored. Clone size was classified as small (1-100 cells), medium (101-30 000 cells), or large (30 000 to < 150 000; n = 192-288). Cell death, defined by the percentage of wells with dead cells, was not significantly different from control conditions (GFs only) on the compound treatment in any of the tested cell populations (Welch t test LSK+Li, P= .8; LSK+VPA = .13; LSK+Li + VPA, P=.13). (C) Effects of the adding compounds on single-cell colony-plating efficiency of distinct myeloid progenitors. Data are shown as the percentage of cells with colony-forming ability. (D) Ability of distinct myeloid progenitors to generate secondary CFU-GM colonies derived from a single cell (n = 20-48). The bars represent the percentage of single cell–derived primary colonies that gave rise to secondary CFU-GM colonies. The individual colors in the bars indicate the number of secondary CFU-GM colonies derived from the single cell. The differences between groups were evaluated by Welch *t* test, **P* <.05, ***P* <.01, and ****P* <.001.

activity of these progenitor populations, resulting in ~60% and ~35% of secondary colonies, respectively. The addition of Li to primary or secondary single-cell cultures did not affect cell (re)plating efficiencies (Figure 4D).

Summarizing, the effect of VPA or the combination of VPA+Li on colony-forming ability varied, depending on the differentiation status of the cell. VPA and VPA+Li inhibited

proliferation and colony-forming ability of LSK cells but increased its self-renewal potential measured by replating. Although proliferation and clonogenic activity of CMPs were not significantly influenced, VPA and VPA+Li induced replating capacity of this population. GMPs were specifically sensitive to VPA or the combination treatment, because the compounds not only strongly increased clonogenic activity, plating efficiency, and proliferation of this population but also induced their self-renewal capacity.

VPA and Li synergistically preserve HSPC gene expression profile. To identify molecular events by which VPA and/or Li are able to inhibit differentiation, we performed expression arrays on 7-day differentiated LSK cells in the presence or absence of VPA with or without Li. RNA samples were harvested at the time when *in vitro* and *in vivo* assays were initiated. Differential expression analyses (for details, see "Gene expression analysis") showed distinct effects of Li and VPA on gene expression. Although no differentially expressed genes could be identified on Li treatment compared with GFs only (Figure 5A), VPA significantly affected 60 genes, of which 10 were up-regulated and 50 down-regulated. Strikingly, an obvious more than additive effect was observed when Li was added to VPA in our differentiation cultures. The combined treatment of VPA+Li resulted in differential expression of 360 genes (110 up-regulated and 250 down-regulated), showing a strong synergistic effect (Figure 5A). The lists of all genes differentially expressed on the compounds treatment is presented in supplemental Table 1. To assess whether VPA+Li treatment inhibited differentiation by preserving a primitive cell transcriptome, the changes in expression of VPA+Li targets (Figure 5A and supplemental Table 1) were compared with normal hematopoietic differentiation. For this purpose, previously obtained transcriptome data of 4 developmentally distinct hematopoietic cell stages, stem cells ($Lin^-Sca1^+ckit^+$), progenitors ($Lin^-Sca1^-ckit^+$), myeloid ($Gr1^+$), and erythroid ($Ter119^+$) cells, were used.[29] Analysis found that target genes that were up-regulated by VPA+Li were more abundantly expressed in primary stem/progenitor cells compared with differentiated myeloid and erythroid cells, whereas VPA+Li down-regulated targets showed the opposite pattern, being higher expressed in differentiated than in uncommitted cells. (Figure 5B) Since VPA+Li treatment altered expression of 360 genes, an important question is whether expression changes of these targets are all independent or co-regulated. Cellular differentiation is most probably orchestrated by networks of co-regulated genes, rather than by changes in expression of individual genes. To address this question, the method described in detail by Voy et al[30] was used. First, gene correlation studies (using Pearson coefficient) were performed with the same transcriptome data of the 4 developmentally distinct hematopoietic cell stages as used for determining cell type expression abundance of VPA+Li affected targets. The strongest correlations, most probably reflecting biologic relevance, were defined by less than -0.8 or > 0.85 thresholds and visualized with Gephi Version 0.8 beta software.[31] Such a gene relevance network, reflecting similarity measures of gene expressions in 4 cell populations, is presented in Figure 5C. VPA+Li targets were color-coded according to the expression abundance. Interestingly, it appeared that VPA+Li targets are co-regulated during normal hematopoietic differentiation, creating high connectivity networks. In addition, genes clustered together according to a specific cell type, creating a network consisting of 3 apparent clusters. One combined cluster contained genes most abundantly expressed in either stem (green) or

Table 1. Expression changes of transcriptional regulators (TRs) affected by VPA and/or Li treatment

TR	VPA	VPA+Li
Hmgn3	+3	+3.32
Gfi1		+2.26
HoxA7	+2.58	+2.22
Prmt5		+2.08
Six1		+1.81
Rbbp7		+1.74
Satb1		+1.69
Wdr77		+1.66
Gata2		+1.64
Phf5a		+1.60
Psmc3ip		1+1.55
Egr2	-3.17	-3.74
Egr1		-3.64
Dab2		-3.52
Il1b	-2.61	-3.02
Bhlhb2		-2.74
Irf5	-2.73	-2.73
Nfkbia		-2.62
Tcf7l2		-2.61
Atf3		-2.48
Bcl6		-2.37
Osm		-2.34
Klf4		-2.28
Junb		-2.14
Inhba		-1.98
S100a1		-1.91
Irf4		-1.90
Klf6		-1.90
Zfhx3		-1.82
Tlr4		-1.82
Irak2		-1.74
Prmt2		-1.72
Hlx		-1.71
Pparg		-1.69
App		-1.62
Mmp14	-2.54	
Tnni2	-1.94	
Tob1	-1.55	

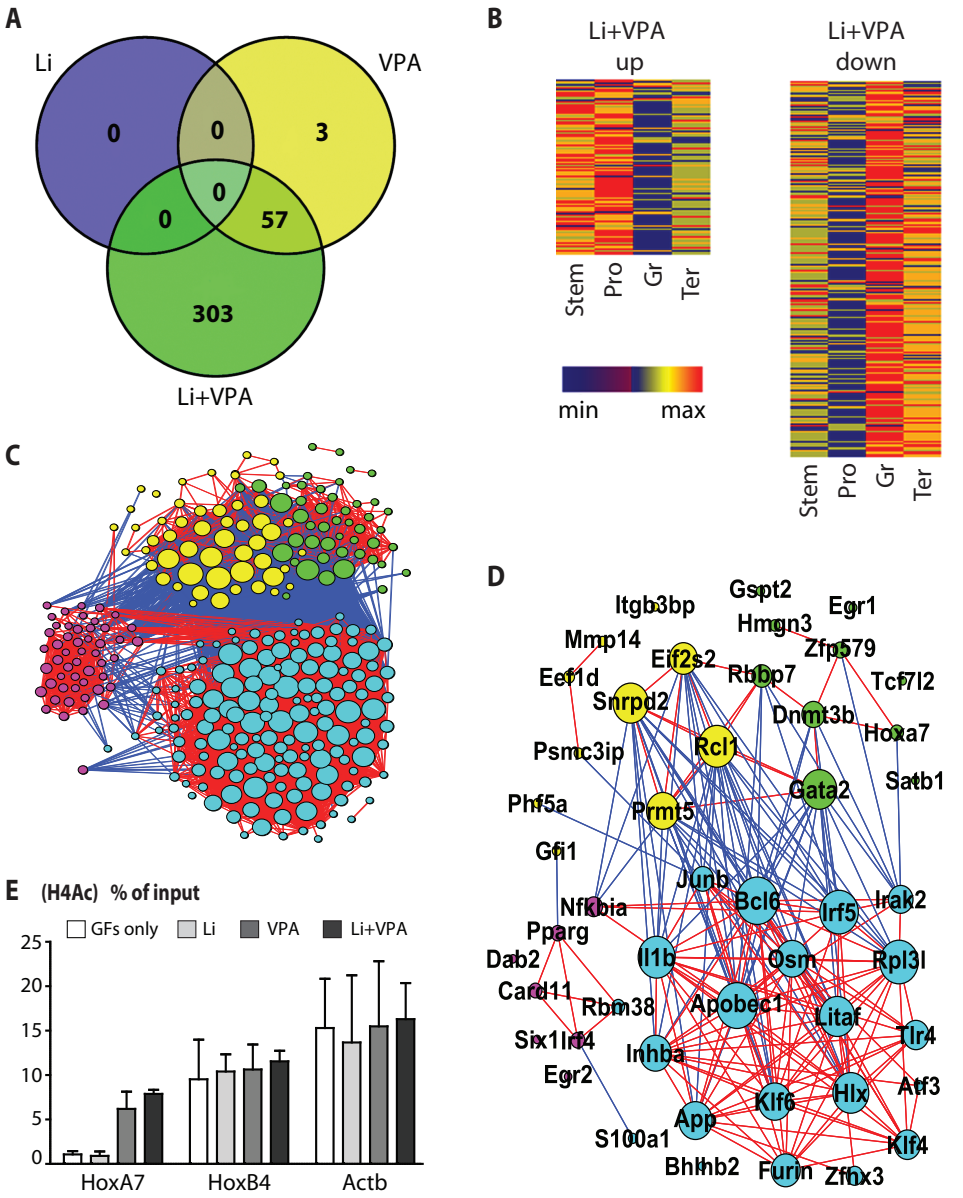

Figure 5. Stem/progenitor cell gene expression profile synergistically preserved by VPA+Li. (A) Venn diagram representation of significantly differentially expressed genes on Li, VPA, or VPA+Li treatment. Venn diagram were created with Venny. (B) Rank-based heat maps of expression levels of genes up-regulated or down-regulated by VPA+Li in 4 freshly isolated cell types, stem (LSK), progenitor ($L^-S^-K^+$), myeloid ($Gr1^+$), and erythroid ($Ter119^+$) cells. Heat maps were created with Genesis Version 1.7.6 software.[50] (C) Graphic representation of gene networks affected by VPA+Li. For similarity measure, Pearson correlation was used with a threshold of > 0.85 (FDR $P < .1$).[30] The network consists of nodes (genes) and edges (biologic relations between nodes). Size of nodes corresponds to amount of connection of a particular node with other nodes. Nodes are color-coded according to their expression abundance in 4 distinct cells types, green nodes refer to genes that are preferentially expressed in stem cells (LSK), yellow nodes shows genes active in ▶

progenitor (yellow) cells, and 2 separate clusters contained genes predominantly expressed in myeloid (blue) or erythroid (purple) cells (Figure 5C). Network analysis found also differential gene induction or repression by the treatment. As expected, genes within a cluster, usually expressed in the same cell type, showed positive correlations (red lines) with each other, suggesting that expression levels of these genes are changed in parallel by VPA+Li. However, strong negative correlations (blue lines) were apparent between genes belonging to separate clusters. Almost all genes within the stem/progenitor cluster displayed inverse correlation with genes within myeloid and erythroid clusters, indicating interesting gene relations between uncommitted and differentiated cells. Collectively, our analyses show that VPA+Li affected the differentiation program of the cell by perturbing the coordinated expression of multiple genes in co-regulated fashion, preserving stem/progenitor genes, and down-regulating differentiation-associated genes.

To try to identify primary candidates probably involved in cell fate decisions, the VPA+Li target list was filtered for transcription factors (TFs) and other DNA and RNA interacting genes. Table 1 provides a full list of transcriptional regulators that were affected by the treatment. A reduced network retained the 3-cluster structure with strong negative correlations between stem/progenitor and differentiated-cell associated TFs (Figure 5D). Among VPA+Li up-regulated targets were several known stem/progenitor TFs, such as *Gata2*, *Gfi-1*, and *HoxA7*.[33-35] Other TFs, with still unknown functions in HSCs, could also be assigned to the stem cell cluster, including *Rbbp7* and *Phf5*. Moreover, TFs involved in epigenetic events, such as *Hmgn3* and *Prmt5*, were up-regulated by the VPA+Li treatment.[36] In contrast, most of the down-regulated targets were correlated with cellular differentiation, including known TFs for myeloid (*Erg1/2*, *Junb*),[37,38] erythroid/megakaryocyte (*Dab2*),[39] and lymphoid (*Irf5*, *Nfkbia*)[40,41] differentiation. TFs up-regulated by the treatment, showing a negative correlation with differentiation-associated TFs, seem to be especially interesting as targets for differentiation-preventing agents.

Because VPA is an epigenetic modifier, known to affect gene expression by preventing chromatin deacetylation, we tested whether the observed changes in gene expression were caused by altered histone acetylation levels. To this end, ChIP with the use of Abs against acetylated histone H4 were performed. As proof of principle, *HoxA7*, up-regulated by both VPA and VPA+Li, was selected for ChIP assay. Interestingly, several reports have suggested that another homeobox gene, *HoxB4*, is a target of VPA in human cells.[16-18] In our study, expression of *HoxB4* was not affected by the compound treatment. The ChIP experiments showed that VPA caused hyperacetylation of histone H4 at regulatory sites of the *HoxA7* promotor, but not of the *HoxB4* promotor. This correlates with the 2.3- and 8-fold higher *HoxA7* expression measured by microarray and quantitative RT-PCR (not shown), respectively, and lack of change

▶ progenitors ($L^-S\text{-}K^+$), blue nodes are prevalent in mature myeloid cells ($Gr1^+$), and purple nodes are expressed in mature erythroid cells ($Ter119^+$). Lines represent correlations of particular genes with others, blue lines represent negative correlations, and red lines indicate positive correlations. Nodes with <1 connection were removed. (D) TF networks significantly changed by VPA+Li treatment. This graph shows essentially the same data as those in panel C, but now only for transcriptional regulators. All network visual representation were performed with Gephi Version 0.8 beta software.[31] (E) Acetylation status of histone H4 at promoter regions of *HoxA7* and *HoxB4* genes. β-actin was used as a housekeeping gene control for ChIP experiments.

in *HoxB4* expression. As expected, Li did not affect the epigenetic status of promotor regions of tested genes (Figure 5F). This indicates that VPA affected expression of *HoxA7* by increasing histone acetylation on regulatory regions of the gene.

Taken together, genome-wide gene expression profiling of VPA- and Li-treated cells showed the synergistic effects of these 2 compounds at the molecular level. Gene relevance networks indicated that these effects required simultaneous changes in multiple genes to prevent differentiation, which was achieved by enhancing genes involved in cell primitiveness and repressing genes associated with cellular differentiation.

DISCUSSION

To date, attempts to define culture conditions that would favor stem cell self-renewal over differentiation have resulted in limited success. We hypothesized that combinations of cytokines and small molecules might represent an integrative approach to prevent differentiation in culture. In this report, we tested the combination of a well-known HDI, VPA, with the simple cation, Li, on HSPC maintenance under strong differentiation pressure and showed that the combination of these 2 compounds displayed strong anti-differentiation effects on hematopoietic differentiation at the biologic and the molecular levels. Although Li as a single agent displayed limited effects on HSPC differentiation, VPA and the combination of VPA+Li were able to preserve *in vitro* functionality of LSK and induced self-renewal of committed progenitors (CMPs and GMPs). Strikingly, after a 7-day differentiation culture only the combination of these 2 agents provided radioprotection to lethally irradiated recipients, shortened the time required for platelet and red blood cell recovery, and enhanced *in vivo* repopulation potential. These observations are consistent with previous reports, suggesting that VPA or Li, can affect fate decision of HSPCs,[16-18,20-22,25,26] but to our knowledge this is the first report to show that VPA+Li act synergistically to delay HSPC differentiation.

In accordance with the biologic data, the most compelling evidence that VPA+Li act synergistically to inhibit LSK differentiation was provided by genome-wide gene expression analysis. Strikingly, VPA+Li affected expression of 360 genes, whereas VPA alone affected only 60 genes, and Li did not affect any genes. Network analysis provided evidence that most genes affected by VPA+Li were co-regulated during normal hematopoietic differentiation. In addition, VPA+Li preserved expression of stem cell-related genes, which coincided with repression of genes involved in differentiation. This indicates that the anti-differentiation effects of VPA+Li are caused by coordinated changes in multiple co-regulated genes, rather than in individual genes. Our findings were further illustrated by the fact that TFs (probably being the primary targets in the network) favoring self-renewal (eg, *Gata2*, *Gfi-1*, *HoxA7*)[33-35] were increased, and differentiation-associated TFs were decreased on VPA+Li treatment. It has been suggested that competition and balance between TFs, such as *Gata2*, *PU.1*, *Gfi-1*, *Erg1/2*, may regulate switches between self-renewal and differentiation and triggers the determination of cell fate.[3,37,42-44] The inhibition of the differentiation program was shown for multipotent uncommitted and also for committed cells, which are at various stages of differentiation. Strikingly, on addition of VPA and VPA+Li, individual CMP and GMP progenitor cells re-acquired replating capacity

in methylcellulose assays, suggesting possible cellular de-differentiation. However, whether the same mechanism is responsible for the effects of Li+VPA on committed progenitor differentiation versus LSK differentiation remains to be determined.

The balance between histone acetylation and deacetylation is thought to play an important role in maintaining chromatin structure consistent with the stem cell state. Epigenetic regulation is believed to be important for cell fate decision, and epigenetic transcriptional repression was suggested to be required for silencing of stem cell genes and subsequent cell differentiation.[14,15,44] VPA has been shown to inhibit HDACs and therefore could interfere with the epigenetic status of the cell.[8] We confirmed increased histone acetylation at the *HoxA7* promotor region, correlating with the increased expression of *HoxA7*. In addition, other confirmed VPA+Li targets (*Gata2*) have been suggested to bind to HDAC or to be involved in epigenetic modifications (*Hmgn3*, *Prmt5*).[36,45] Moreover, the chromatin conformation maintained by VPA might allow a spectrum of Li targets to be better accessible. Thus, common targets of VPA and Li (eg, Wnt pathway)[46] in combination with more accessible chromatin conformation may result in the observed synergistic effect of these 2 compounds. Therefore, modulation of epigenetic regulation of gene expression, as shown here with VPA, can lead to coordinated preservation or up-regulation of genes involved in stem cell maintenance and simultaneous down-regulation of differentiation-associated genes. The resemblance with expression patterns during natural differentiation suggests a physiologic mechanism driving expression of multiple genes determining cell fate. Self-renewal and differentiation seem to be strongly co-dependent, thus *ex vivo* stem cell culturing systems should target both processes to maintain uncommitted cells.

Methods to inhibit stem cell differentiation may be of benefit in *ex vivo* cell therapy protocols, which favor *in vitro* HSPC self-renewal rather than commitment and differentiation. So far, culturing systems with "classic" hematopoietic GFs resulted in stem cell loss because of profound differentiation.[1,2] Recently, factors other than classic hematopoietic GFs (eg, RA, VPA, FGFs, SR1, pleiotropin)[9,10,18,47,48] showed a promising potential in *ex vivo* expansion protocols. Our study underlines that combinatorial screening of Li and VPA and other new compounds may uncover possible additive and/or synergistic effects to modulate HSPC self-renewal and differentiation and thereby may lead to novel approaches in *ex vivo* stem cells culturing systems.

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AUTHORSHIP

Contribution: M.A.W., G.d.H., and R.v.O. designed research; M.A.W., V.v.d.B., S.O., A.A., and M.R. performed research; M.A.W, L.B., V.v.d.B., and G.H. analyzed and interpreted data; and M.A.W. wrote the manuscript with contribution from L.B., G.d.H., and R.v.O.

Conflict-of-interest disclosure: The authors declare no compet- ing financial interests.

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REFERENCES

1. Sauvageau G, Iscove NI, and Humphries RK. *In vitro* and *in vivo* expansion of hematopoietic stem cells. *Oncogene*. 2004;23:7223-7232.
2. Sorrentino BP. Clinical strategies for expansion of haematopoietic stem cells. *Nat Rev Immunol*. 2004;4:878-888.
3. Zon LI. Intrinsic and extrinsic control of haematopoietic stem-cell self-renewal. *Nature*. 2008;453:306-312.
4. Antonchuk J, Sauvageau G, Humphries RK. HOXB4-Induced Expansion of Adult Hematopoietic Stem Cells Ex Vivo. *Cell*. 2002;109:39-45.
5. Rizo A, Dontje B, Vellenga E, de Haan G, Schuringa JJ. Long-term maintenance of human hematopoietic stem/progenitor cells by expression of BMI1. *Blood*. 2008;111:2621-2630.
6. Reya T, Duncan AW, Ailles L, et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature*. 2003;423:409-414.
7. Schugar RC, Robbins PD, and Deasy BM. Small molecules in stem cell self-renewal and differentiation. *Gene Therapy*. 2008;15:126-135.
8. Lyssiotis CA, Lairson LL, Boitano AE, Wurdak H, Zhu S, Schultz PG. Chemical Control of Stem Cell Fate and Developmental Potential. *Angew. Chem. Int. Ed.*. 2011;50:200-242.
9. Purton LE, Bernstein ID, Collins SJ. All-trans retinoic acid enhances the long-term repopulating activity of cultured hematopoietic stem cells. *Blood*. 2000;95:470-477.
10. Boitano AE, Wang J, Romeo R, et al. Aryl Hydrocarbon Receptor Antagonists Promote the Expansion of Human Hematopoietic Stem Cells. *Science*. 2010;329:1345-1348.
11. Huangfu D, Osafune K, Maehr R, et al. Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. *Nat Biotech*. 2008;26:1269-1275.
12. Botrugno OA, Santoro F, Minucci S. Histone deacetylase inhibitors as a new weapon in the arsenal of differentiation therapies of cancer. *Cancer Lett*. 2009;280:134-144.
13. Focosi D, Azzara A, Kast RE, Carulli G, Petrini M. Lithium and hematology: established and proposed uses. *J Leukoc Biol*. 2009;85:20-28.
14. Zardo G, Cimino G, Nervi C. Epigenetic plasticity of chromatin in embryonic and hematopoietic stem//progenitor cells: therapeutic potential of cell reprogramming. *Leukemia*. 2008;22:1503-1518.
15. Meissner A. Epigenetic modifications in pluripotent and differentiated cells. *Nat Biotech*. 2010;28:1079-1088.
16. Bug G, Gül H, Schwarz K, et al. Valproic Acid Stimulates Proliferation and Self-renewal of Hematopoietic Stem Cells. *Cancer Res*. 2005;65:2537-2541.
17. De Felice L, Tatarelli C, Mascolo MG, et al. Histone Deacetylase Inhibitor Valproic Acid Enhances the Cytokine-Induced Expansion of Human Hematopoietic Stem Cells. *Cancer Res*. 2005;65:1505-1513.
18. Seet L, Teng E, Lai Y, et al. Valproic acid enhances the engraftability of human umbilical cord blood hematopoietic stem cells expanded under serum-free conditions. *Eur J Haematol*. 2009;82:124-132.
19. Milhem M, Mahmud N, Lavelle D, et al. Modification of hematopoietic stem cell fate by 5aza 2'deoxycytidine and trichostatin A. *Blood*. 2004;103:4102-4110.
20. Yamamura K, Ohishi K, Katayama N, et al. Pleiotropic role of histone deacetylases in the regulation of human adult erythropoiesis. *Br J Haematol*. 2006;135:242-253.
21. Bartels M, Geest CR, Bierings M, Buitenhuis M, Coffer PJ. Histone deacetylase inhibition modulates cell fate decisions during myeloid differentiation. *Haematol*. 2010;95:1052-1060.
22. Chateauvieux S, Eifes S, Morceau F, et al. Valproic acid perturbs hematopoietic homeostasis by inhibition of erythroid differentiation and activation of the myelo-monocytic pathway. *Biochem Pharmacol*. 2011;81:498-509.
23. Özdemir MA, Sofuoglu S, Tanrikulu G, Aldanmaz F, Esel E, Dündar S. Lithium-induced hematologic changes in patients with bipolar affective disorder. *Biol Psychiatry*. 1994;35:210-213.
24. Ballin A, Lehman D, Sirota P, Litvinjuk U, Meytes D. Increased number of peripheral blood CD34+ cells in lithium-treated patients. *Br J Haematol*. 1998;100:219-221.
25. 'Gallicchio V',MG'. Modulation of murine pluripotential stem cell proliferation in vivo by lithium carbonate. *Blood*. 1980;56:1150-1152.
26. Quesenberry P, Coppola M, Gualtieri R, et al. Lithium stimulation of murine hematopoiesis in liquid culture: an effect mediated by marrow stromal cells. *Blood*. 1984;63:121-127.

27. Phiel CJ and Klein PS. Molecular targets of lithium action. *Annu Rev Pharmacol Toxicol*. 2001;41:789-813.
28. van Os RP, Dethmers-Ausema B, and de Haan G. In Vitro Assays for Cobblestone Area-Forming Cells, LTC-IC, and CFU-C. *Meth Mol Biol*. 2008;430:143-157.
29. Gerrits A, Li Y, Tesson BM, et al. Expression Quantitative Trait Loci Are Highly Sensitive to Cellular Differentiation State. *PLoS Genet*. 2009;5:e1000692.
30. Voy BH, Scharff JA, Perkins AD, et al. Extracting Gene Networks for Low-Dose Radiation Using Graph Theoretical Algorithms. *PLoS Comput Biol*. 2006;2:e89.
31. Bastian, M., Heymann, S., Jacomy,M. Gephi: An Open Source Software for Exploring and Manipulating Networks. 2009. *International AAAI Conference on Weblogs and Social Media*
32. Frank SR, Schroeder M, Fernandez P, Taubert S, Amati B. Binding of c-Myc to chromatin mediates mitogen-induced acetylation of histone H4 and gene activation. *Genes Dev*. 2001;15:2069-2082.
33. Tsai FY, Keller G, Kuo FC, et al. An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature*. 1994;371:221-226.
34. Zeng H, Yucel R, Kosan C, Klein-Hitpass L, Moroy T. Transcription factor Gfi1 regulates self-renewal and engraftment of hematopoietic stem cells. *EMBO J*. 2004;23:4116-4125.
35. Ayton P and andCleary M. Transformation of myeloid progenitors by MLL oncoproteins is dependent on *Hoxa7* and *Hoxa9*. *Genes Dev*. 2003;17:2298-2307.
36. Roloff TC and Nuber UA. Chromatin, epigenetics and stem cells. *Eur J Cell Biol*. 2005;84:123-135.
37. Laslo P, Spooner CJ, Warmflash A, et al. Multilineage Transcriptional Priming and Determination of Alternate Hematopoietic Cell Fates. *Cell*. 2006;126:755-766.
38. Passegué E, Wagner EF, Weissman IL. JunB Deficiency Leads to a Myeloproliferative Disorder Arising from Hematopoietic Stem Cells. *Cell*. 2004;119:431-443.
39. Tseng C, Huang C, Tseng C, Lin M, Hsieh J, Tseng C. Induction of Disabled-2 Gene during Megakaryocyte Differentiation of K562 Cells. *Biochem Biophys Res Commun*. 2001;285:129-135.
40. Lien C, Fang C, Huso D, Livak F, Lu R, Pitha PM. Critical role of IRF-5 in regulation of B-cell differentiation. *PNAS*. 2010;107:4664-4668.
41. Goudeau B, Huetz F, Samson S, et al. IκBα/IκBε deficiency reveals that a critical NF-κB dosage is required for lymphocyte survival. *PNAS*. 2003;100:15800-15805.
42. Walsh JC, DeKoter RP, Lee H, et al. Cooperative and Antagonistic Interplay between PU.1 and GATA-2 in the Specification of Myeloid Cell Fates. *Immunity*. 2002;17:665-676.
43. Wilson NK, Foster SD, Wang X, et al. Combinatorial Transcriptional Control In Blood Stem/Progenitor Cells: Genome-wide Analysis of Ten Major Transcriptional Regulators. *Cell Stem Cell*. 2010;7:532-544.
44. Pimanda JE and Göttgens B. Gene regulatory networks governing haematopoietic stem cell development and identity. *Int J Dev Biol*. 2010;54:1201-1211.
45. Ozawa Y, Towatari M, Tsuzuki S, et al. Histone deacetylase 3 associates with and represses the transcription factor GATA-2. *Blood*. 2001;98:2116-2123.
46. Gurvich N and Klein PS. Lithium and valproic acid: parallels and contrasts in diverse signaling contexts. *Pharmacol Ther*. 2002;96:45-66.
47. de Haan G, Weersing E, Dontje B, et al. In Vitro Generation of Long-Term Repopulating Hematopoietic Stem Cells by Fibroblast Growth Factor-1. *Dev Cell*. 2003;4:241-251.
48. Himburg HA, Muramoto GG, Daher P, et al. Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells. *Nat Med*. 2010;16:475-482.
49. Oliveros J. VENNY. An interactive tool for comparing lists with Venn diagrams. *BioinfoGP, CNB-CSIC*. 2007.
50. Sturn A, Quackenbush J, Trajanoski Z. Genesis: cluster analysis of microarray data. *Bioinformatics*. 2002;18:207-208.

SUPPLEMENTAL INFORMATION

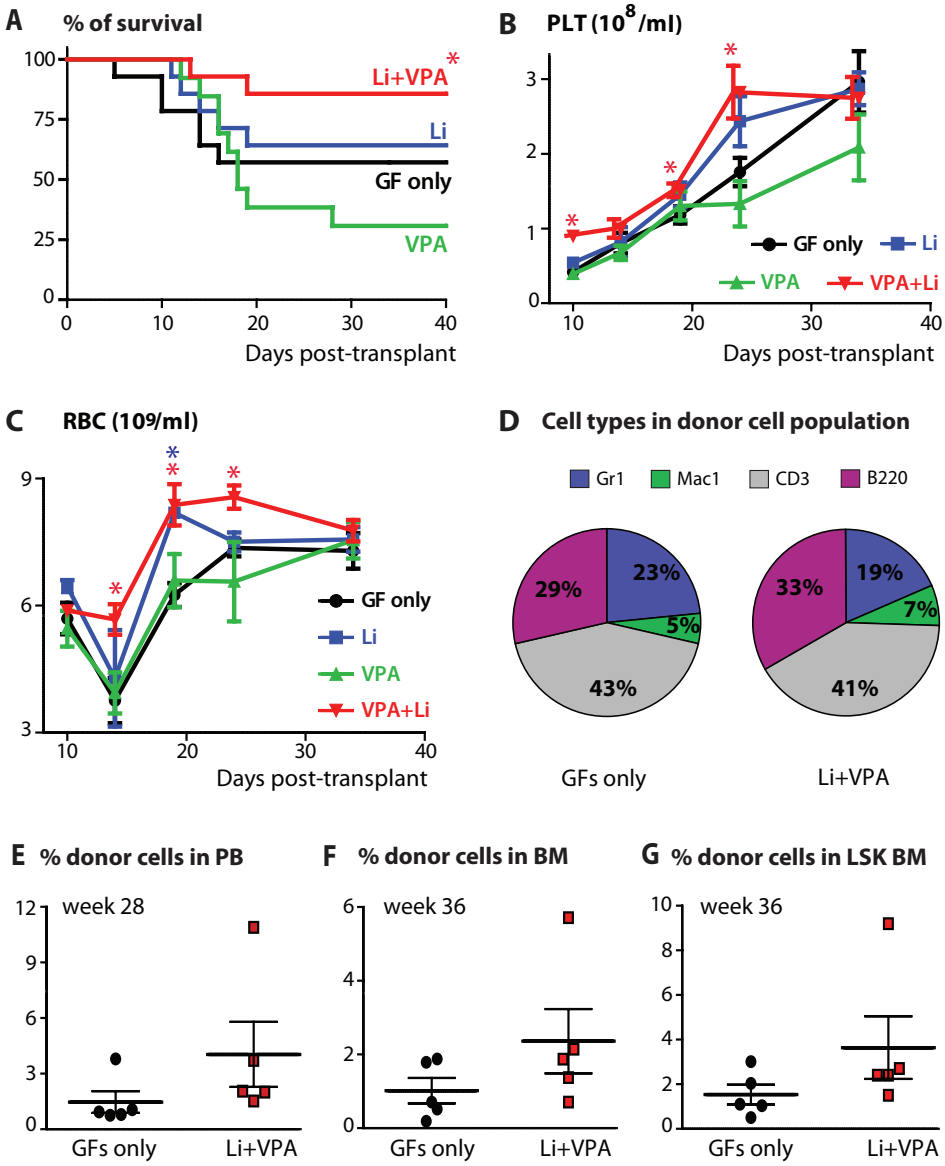

Figure S1. Effects of VPA and/or Li treatment on radioprotection and long-time repopulation ability of BM cells. LSK cell cultured for 7 days with GM-CSF and SCF in the presence or absence of VPA and/or Li were transplanted into lethally irradiated recipients with or without competitors. (A) Survival of the recipients was screened for 40 days following non-competitive transplantations. Significance was calculated using Mantel-Cox test (p=0.043) and Gehan-Breslow-Wilcoxon test (p=0.0394). (B) Platelet counts in the peripheral blood of recipient mice at 10 up to 40 days post-transplantation (Non-competitive transplantations). Error bars represent standard error of the mean. (C) Red blood cell counts in the peripheral ▶

Supplemental information available online on request

Table S1. Gene expression dataset on GFs, Li, VPA, and VPA+Li- treated cells.
(http://bloodjournal.hematologylibrary.org/content/119/13/3050/suppl/DC1)

▶ blood of recipient mice at 10 up to 40 days after transplantation (Non-competitive transplantations). Error bars represent standard error of the mean. (D) The blood cell type distribution presented as percentage of distinct mature cells type contribution into the donor blood cell population at 28 weeks post-transplantation (competitive transplantations). (E) Percentage of donor cells in the peripheral blood of individual recipient mice at 28 weeks post-transplantation (competitive transplantations, the same as on figure 4C). Error bars represent standard error of the mean (n=5). (F) Percentage of donor cells in the bone marrow of individual recipient mice at 36 weeks post-transplantation (competitive transplantations). Error bars represent standard error of the mean (n=5). (G) Percentage of donor cells in the LSK fraction of bone marrow of individual recipient mice at 36 weeks post-transplantation (competitive transplantations). Error bars represent standard error of the mean (n=5). The differences between groups were evaluated by Welch's t test, $^{*}P<.05$.

CHAPTER 5

SCA-1 IS AN EARLY-RESPONSE TARGET OF HISTONE DEACETYLASE INHIBITORS AND MARKS HEMATOPOIETIC CELLS WITH ENHANCED FUNCTION

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ABSTRACT

Histone deacetylase inhibitors (HDIs) have been shown to enhance hematopoietic stem and progenitor cell activity and improve stem cell outcomes after *ex vivo* culture. Identification of gene targets of HDIs is required to understand the full potential of these compounds and can allow for improved stem cell culturing protocols. The molecular process that underlies the biological effects of valproic acid (VPA), a widely used HDI, on hematopoietic stem/progenitor cells was investigated by studying the early-response genes of VPA. These genes were linked to VPA-induced enhancement of cell function as measured by *in vitro* assays. Genome-wide gene expression studies revealed over-representation of genes involved in glutathione metabolism, receptor and signal transducer activity, and changes in the hematopoietic stem/progenitor cells surface profile after short, 24-hour VPA treatment. Sca-1, a well-known and widely used stem cell surface marker, was identified as a prominent VPA target. We showed that multiple HDIs induce Sca-1 expression on hematopoietic cells. VPA strongly preserved Sca-1 expression on $Lin^{-}Sca1^{+}ckit^{+}$ cells, but also reactivated Sca-1 on committed progenitor cells that were $Sca\text{-}1^{neg}$, thereby reverting them to the $Lin^{-}Sca1^{+}ckit^{+}$ phenotype. We demonstrated that reacquired Sca-1 expression coincided with induced self-renewal capacity as measured by *in vitro* replating assays, while Sca-1 itself was not required for the biological effects of VPA as demonstrated using Sca-1-deficient progenitor cells. In conclusion, our results show that VPA modulates several genes involved in multiple signal transduction pathways, of which Sca-1 was shown to mark cells with increased self-renewal capacity in response to HDIs.

INTRODUCTION

Hematopoietic stem cells (HSCs) are characterized by their self-renewal capacity and ability to generate all mature blood cells via a well-defined differentiation cascade. Hematopoietic lineage specification occurs in a stepwise process of commitment that eventually leads to formation of myeloid and lymphoid lineages.[1,2] Although HSCs differentiate and progress down the hematopoietic hierarchy, their multipotency, self-renewal, and lineage choice become progressively and irreversibly restricted.[3,4] However, identification of mixed lympho-myeloid progenitors suggests that some overlap between these two lineages does exist.[5,6] Lineage restriction can be overcome to some extent by ectopic expression of key lineage-specific transcription factors, for example, overexpression of myeloid transcription factors PU.1 or CEBPα in differentiated lymphoid cells resulted in transdifferentiation of these cells into macrophages, whereas overexpression of GATA-1 caused switching from myeloid to erythroid lineage.[7-9] Hematopoietic stem and progenitor cells can be identified by a specific cell surface marker profile, by the capacity of dye efflux, or on the basis of their metabolic properties.[10] In combination with other markers, Sca-1 is the most commonly used cell surface marker to enrich for adult murine HSCs. The Ly-6a gene, more widely known as Sca-1, encodes for a glycosyl phosphatidylinositol-anchored cell surface protein of the Ly6 family and is one of the early markers expressed on emerging HSC.[11] During adulthood, Sca-1 is present on both primitive and more differentiated cell subsets, suggesting complex regulation during hematopoietic ontology.[12] Sca-1 expression phenotypically separates the stem cell compartment from committed myeloid progenitors, and its rapid down-regulation has been shown to be crucial for myeloid differentiation.[13] Transplantations with purified cells and gene knock-in reporter studies have indisputably shown the utility of Sca-1 as an HSC marker.[14-16] In addition, studies with Sca-1 knockout (KO) mice suggest that Sca-1 is not only a stem cell marker, but can also regulate the developmental program of HSCs and progenitor populations.[13,17] Although there has been much speculation about the putative Sca-1 function and mechanism of its action, the role of this marker in HSCs remains enigmatic. Previously, we reported on the maintenance of Sca-1 (and c-kit) expression on hematopoietic stem/progenitor cells (HSPCs) and preservation of their functional potential after long-term *in vitro* treatment with valproic acid (VPA), a histone deacetylase inhibitor (HDI).[18] Additionally, VPA has been shown to display an important biological effect on HSCs and their committed progeny.[18-23] HDIs can affect gene expression by preventing histone deacetylation and modifying the chromatin structure at regulatory loci of the gene. Importantly, HDIs including VPA have been used in leukemia as a differentiation therapy. Therefore, it is crucial to understand the full spectrum of HDI-related effects. The molecular process that underlies the biological effects of HDIs can be illuminated by detecting HDI-responsive genes. In a previous report, we determined molecular consequences of long-term VPA treatment and we showed that this HDI preserved HSPC function and gene expression profile after a 7-day culture. Here, we performed microarrays after short-term, 24-hour VPA stimulation to identify early-response VPA targets in HSPCs. Our data provide the first analysis of direct VPA targets in primitive hematopoietic cells and show that Sca-1 is a faithful marker to identify HDI-responsive cells after *in vitro* culture.

MATERIAL AND METHODS

Mice. Female wild-type C57BL/6 mice (Harlan, Horst, The Netherlands) or Sca-1-deficient C57BL/6 mice (Sca-1 KO mice) were used as a source of hematopoietic cells. Sca-1 KO mice were originally generated by William Stanford, University of Toronto, Canada and were kindly provided to us by Marieke Essers and Andreas Trumpp, Heidelberg Institute for Stem Cell Technologies and Experimental Medicine (Heidelberg, Germany). The local animal ethical committee of the University of Groningen approved all animal experiments.

Isolation of hematopoietic cell populations. Mice were anesthetized and sacrificed by cervical dislocation. Unfractionated bone marrow cells were obtained by crushing femora, tibiae, and pelvic bones. Cell suspensions were filtered through a 100-µm cell strainer (BD Biosciences) to remove debris and the cells were counted on a Medonic CA620 analyzer (A.Menarini Diagnostics). After erythrocyte lysis, cells were stained with lineage cocktail (i.e., A700-Mac1, A700-Gr1, A700-Ter119, A700-CD3, and A700-B220), Pacific Blue-Sca1, phycoerythrin (PE)-ckit, fluorescein isothiocyanate (FITC)-CD34, and PE-Cy7-CD16/32 for isolation of uncommitted and committed cells. For isolation of differentiated cell populations, cells were stained with selected lineage markers, FITC-Ter119, PE-Gr-1, allophycocyanin-(APC)-CD115, PE-Cy7-CD169, and Pacific Blue-Sca-1. Subsequently, cells were resuspended in propidium iodide solution (1 mg/mL) and uncommitted ($Lin^{-}Sca1^{+}ckit^{+}$ [LSK]), committed (common myeloid progenitors [CMP]: $L^{-}S^{neg}K^{+}CD34^{high}CD16/32^{mid}$, granulocyte-macrophage progenitors [GMP]: $L^{-}S^{neg}K^{+}CD34^{high}CD16/32^{high}$, and megakaryocyte-erythroid progenitors [MEP]: $L^{-}S^{neg}K^{+}CD34^{neg}CD16/32^{neg}$), and differentiated hematopoietic cell subpopulations (granulocytes: $Ter119^{neg}B220^{neg}CD3^{neg}Gr1^{high}$ $CD115^{neg}CD169^{neg}$, monocytes: $Ter119^{neg}B220^{neg}$ $CD3^{neg}Gr1^{high}CD115^{high}CD169^{neg}$, macrophages: $Ter119^{neg}B220^{neg}CD3^{neg}Gr1^{mid/neg}CD115^{mid/neg}CD169^{mid/high}$, and erythrocytes: $B220^{neg}CD3^{neg}Gr1^{neg}Ter119^{high}$) were sorted by a MoFlo XDP cell sorter or MoFlo Astrios (Beckman Coulter).[24-26]

Compounds and HDI stimulation. Fluorescence-activated cell-sorted (FACS) uncommitted, committed, and differentiated hematopoietic cell subpopulations were stimulated for 24 hours with a range of HDI concentrations in the presence of 10% fetal calf serum, murine stem cell factor (300 ng/mL; Peprotech), and recombinant murine interleukin-11 (20 ng/mL; R&D Systems). Cells were cultured in StemSpan medium (StemCell Technologies) at 37°C in a humidified atmosphere and 5% CO_2 in air. VPA (Sigma-Aldrich) was dissolved in phosphate-buffered saline solution (PAA Laboratories GmbH), whereas MS-275 (Selleck) and Apicidin (Enzo Life Sciences) were dissolved in dimethyl-sulfoxide and further diluted in phosphate-buffered saline with 0.2% bovine serum albumin (to obtain the desired compound concentration). In case of two latter compounds, control cells were dimethyl-sulfoxide vehicle-treated with adequate dimethyl-sulfoxide concentration.

Gene expression analysis. Gene expression analysis was performed in LSK cells that were cultured for 24 hours in the presence or absence of VPA. All samples were analyzed in independent biological triplicates. Total RNA was isolated using the RNeasy kit (Qiagen), according to manufacturer's protocol. RNA concentration, quality, and integrity were measured using the Experion Automated Electophoresis System (Bio-Rad). RNA was amplified using

the Illumina TotalPrep RNA Amplification Kit (Ambion/Applied Bioscience) and hybridized to Mouse Ref-8_V2 expression platform (Illumina) according to the manufacturer's instruction. Scanning was carried out on the iScan System (Illumina). Image analyses and extraction of raw expression data were performed using BeadStudio software (Illumina) with default settings, no background subtraction, and no normalization. Raw data were thresholded at 1, log2-transformed, and quantile normalized using GeneSpring-GX11.0 (Agilent). From the initial probe list (25,697 probes) probes not expressed in any replicate of the two conditions were excluded. The redefined list (16,050 probes) consisted of only those probes that were flagged as marginal or present. Default detection p value cut-offs of 0.8 for present and 0.6 for absent were used for flags. Probes significantly differentially expressed between VPA-stimulated and control cells were defined based on the three sigma rule.[27,28] First, the mean (standard deviation) of both groups was defined, and second, genes for which the expression difference exceeded 3 standard deviations were selected. For data with normal distribution, this approximately represents $p<0.05$. Subsequently, we applied >1.5-fold cutoff to select biologically relevant probes for differential expression. Finally, a redefined probe list was subjected to overrepresentation analyses using a gene set property analysis tool, Gene Trail.[29] Analyses were performed with the Gene Trail default setting and a manually defined background consisting of all probes present on the murine Illumina Ref_8 arrays used in this study. All raw data were deposited in the NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/), accession number GSE41020.

Sca-1 induction. After 24 hours incubation in the presence or absence of an HDI, distinct cell populations were stained with a cocktail of antibodies as described and analyzed for Sca-1 marker expression by flow cytometry on the LSR-II flow cytometer (BD Bioscience). Alternatively, 24-hour incubated cells were harvested for Sca-1 gene expression analyses. Total RNA was isolated as described, followed by complementary DNA synthesis according to manufacturer's protocol. The coding regions of *Sca-1* gene were amplified by quantitative polymerase chain reaction (Bio-Rad) using gene-specific primer pairs for *Sca-1* (fwd: TGGGTACTAAGGTCAACGTGAAGACTTCC rev: TGGAGGTCATTGGGAGGACCATCAG) and *Gapdh* (fwd: ATGGCCTTCCGTGTTCCTAC rev: GCCTGCTTCACCACCTTCTT).

Functional cell analysis. After 24 hours stimulation with or without VPA, cells were resorted based on Sca-1 expression by a MoFlo XDP cell sorter. Clonogenic potential of Sca-1^{neg} and Sca-1^{pos} cells from HDI stimulated and non-stimulated cultures of wild-type or Sca-1 KO cells was determined by colony-forming unit (CFU) assay. CFU assays were performed as described previously.[18,30]

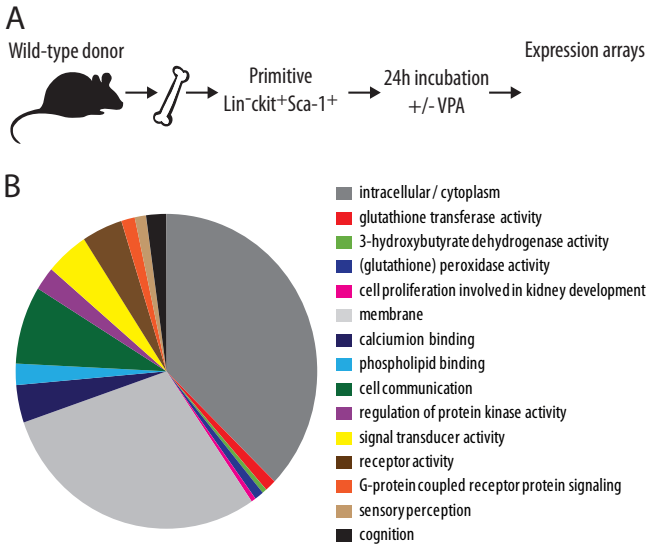
Statistics. BeadStudio and GeneSpring GX 11.5 software were used for analysis of Illumina arrays. Data were further analyzed based on the three sigma rule and Gene Trail software as described. Differences in functionality of cell populations were analyzed using the Mann-Whitney U test (IBM SPSS software, Armonk).

RESULTS

Identification of early VPA-responsive genes. Previously, we demonstrated enhancement of HSPCs function and induction of self-renewal capacity of committed progenitors after

7-day VPA treatment and identified molecular changes induced by a 7-day VPA treatment of HSPCs.[18] However, 7-day stimulation of cells with VPA will lead to a cascade of molecular events that will preclude identification of early VPA targets. To overcome this and to identify early VPA-responsive genes in HSPCs, we analyzed gene expression profiles of LSK cells as early as 24 hours after onset of incubation with or without VPA (Figure 1A). We identified 366 differentially expressed probes (Supplementary Table S1) representing 1.4% of all probes present on the array. The low number of VPA-affected genes is in agreement with previously reported effects of HDIs, suggesting that these compounds affect only a small fraction of the genome.[31-33] Among the differentially expressed probes, 298 probes were up-regulated and 68 probes were down-regulated upon 24 hours VPA stimulation. Because HDIs are expected to prevent deacetylation of histones, we focused on up-regulated genes as primary VPA targets. Among those, transcriptional regulators and genes involved in chromatin remodeling were found, such as *Egr1*, *Rb1*, *Tshz3*, *Bahd1*, or *Hmgn3*. Of these, *Egr1* has been implicated in HSC functioning[34], but the other genes can also play a role in HSC. To functionally assign the VPA affected genes and to test whether any category of genes was over-represented in the set, we subjected the list of 298 up-regulated probes to over-representation analyses according to Gene Ontology categories using Gene Trail. Target genes could be categorized into two main cellular compartments: intracellular/cytoplasmic compartment and membrane compartment. Among the over-represented categories were glutathione metabolism, cell communication, signal transducer activity, and receptor activity (Figure 1B). Glutathione metabolism can be considered as an early cellular response to VPA and mechanism of its detoxification.[35,36] However, we focused on the effects of VPA on the expression of signal transducers and receptors to identify markers on HSPC to detect HDI responsiveness. Over-representation of these categories is clearly prominent because half of the top 12 up-regulated early-response VPA targets (Figure 1C) included genes involved in signal activation and transduction. Among these genes were two widely known and used HSPC markers, *Ly-6a* (*Sca-1*) and *Thy-1*. Sca-1 is included in many hematopoietic staining protocols and is a solid stem cell marker for other adult stem cells as well. We further analyzed Sca-1 as a potential early response VPA target.

VPA (re-)induces Sca-1 expression at various levels of hematopoietic differentiation. To study the effects of VPA on Sca-1 expression during hematopoiesis in more detail, we isolated hematopoietic cells at distinct levels of differentiation. Undifferentiated LSK cells, committed progenitors that are negative for Sca-1 expression (CMPs, GMPs, and MEPs), and Sca-1^{neg} differentiated cells (granulocytes, monocytes, macrophages, and erythrocytes) were incubated for 24 hours with or without VPA. After VPA stimulation, we reanalyzed Sca-1 marker expression on these cells by flow cytometry (Figure 2A). Of note, the concentration used in these experiments did not significantly affect cell numbers during the 24-hour incubation time (data not shown). During 24 hours culture, approximately 23% of purified LSK cells lost Sca-1 marker expression, whereas exposure to VPA strongly prevented this loss, resulting in 98% of cells with preserved Sca-1 expression and maintaining an immature LSK phenotype (Figure 2B). Next, we tested whether Sca-1 could be reactivated in CMPs and GMPs that had lost its expression during differentiation. After 24 hours incubation without VPA, a small percentage of CMP and GMP cells (3% and 0.6%, respectively) up-regulated Sca-1 expression. Strikingly, in the presence of VPA,



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Gene Symbol	+VPA
Ckb	+7.04
Thy1	**+6.49**
Gpc3	+4.28
Fscn1	+3.79
Fbxo2	+3.71
Agrn	+3.65
Gpc1	+3.60
Bex4	+3.45
Tceal5	+3.36
Prnp	+3.29
Ly6a	**+2.91**
Prkcb1	+2.88

Figure 1. Identification of early VPA targets. (A) Experimental design to test effects of 24 hours VPA treatment of LSK cells on gene expression. (B) Functional characterization of genes up-regulated by 24 hours VPA stimulation. List of 298 probes representing 244 genes was subjected to over-representation analyses using GeneTrail according to GeneOntology (GO) categories. Genes assigned to common GO categories were grouped together (Supplementary Table S2; online only). The pie chart represents the significantly over-represented categories. (C) Table showing top 12 up-regulated genes after 24 hours VPA stimulation of LSK cells.

more than 30% of CMP and GMP cells reacquired Sca-1 expression, phenotypically reverting these cells to an LSK phenotype (Figure 2B). Furthermore, Sca-1 could also be reinduced on more differentiated cells, such as subsets of differentiated myeloid cells (FACS-sorted within Sca-1^{neg} gate), including monocytes and macrophages, but not on granulocytes (Figure 2C). No difference in Sca-1 expression could be observed between VPA-stimulated and non-VPA-stimulated erythroid progenitors (MEP) or differentiated erythrocytes (Figure 2B, C). Additionally, Thy-1 marker expression, the other HSPC marker identified as early VPA target, could also be induced on HSPCs after 24 hours stimulation with VPA, but to a lesser extent (data not shown). Together these data confirm the microarray results and show that VPA affects expression of known HSPC markers. Collectively, VPA can not only up-regulate Sca-1 expression on HSPCs that do express this marker, but also can reactivate Sca-1 on cells that have lost its expression in the process of differentiation, reverting CMP and GMP cells to a more immature LSK phenotype. In addition, myeloid cells are particularly sensitive to VPA-induced up-regulation of Sca-1.

Distinct HDIs (re-)induce Sca-1 expression in a dose-related manner. We tested whether the effects of VPA on Sca-1 expression were specific for VPA or were shared by distinct HDIs. To address this question, we selected two additional HDIs, Apicidin and MS-275, which differ from VPA in their chemical structure and specificity. VPA is a short-chain fatty acid pan-inhibitor

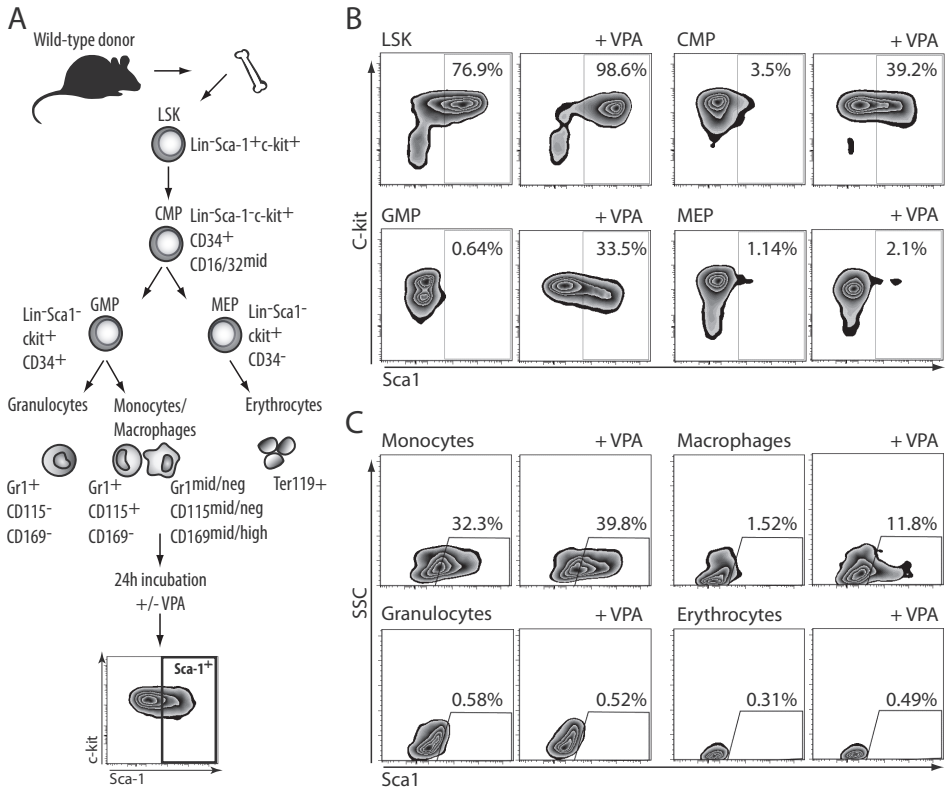

Figure 2. Effects of VPA on Sca-1 expression during hematopoiesis. (A) Experimental design to test the effects of VPA on Sca-1 expression during hematopoietic differentiation. (B) Representative FACS plots demonstrating the effects of 24 hours of VPA stimulation on uncommitted (LSK) and committed (CMP, GMP, MEP) hematopoietic cell populations. Cells on the graph were pre-gated for viable cell population based on SSC, FSC, and propidium iodide (PI) profile. (C) Representative FACS plots demonstrating the effects of 24 hours VPA stimulation on differentiated hematopoietic cell populations (monocytes, macrophages, granulocytes, and erythrocytes). Cells on the graph were pre-gated for viable cell population based on side scatter (SSC), forward scatter, and PI profile.

reported to inhibit activity of both class I and class II histone deacetylase (HDACs). Apicidin, a cyclic tetrapeptide, and MS-275, a benzamide, are HDAC class I-specific inhibitors, with Apicidin being selective for HDAC-2 and -3 and MS-275 showing high selectivity toward HDAC-1 and -3.[37] To assess the effects of these HDIs on Sca-1 expression, we FACS-sorted hematopoietic progenitors defined by the $L^{-}S^{neg}K^{+}$ phenotype and reanalyzed Sca-1 expression on protein and messenger RNA levels after 24 hours stimulation with increasing HDI concentrations (Figure 3A). All three tested HDIs, VPA, Apicidin, and MS- 275, (re-)induced Sca-1 expression on Sca-1^{neg} hematopoietic cells in a concentration-dependent manner, on both protein and messenger RNA levels (Fig. 3B-D). VPA and Apicidin showed similar efficiency to induce Sca-1, resulting in 50% and 40% cells with induced Sca-1, respectively (Figure 3B, C). MS-275, highly selective for HDAC-1, showed the strongest up-regulation of Sca-1 expression, leading to 80% Sca-1^{pos}

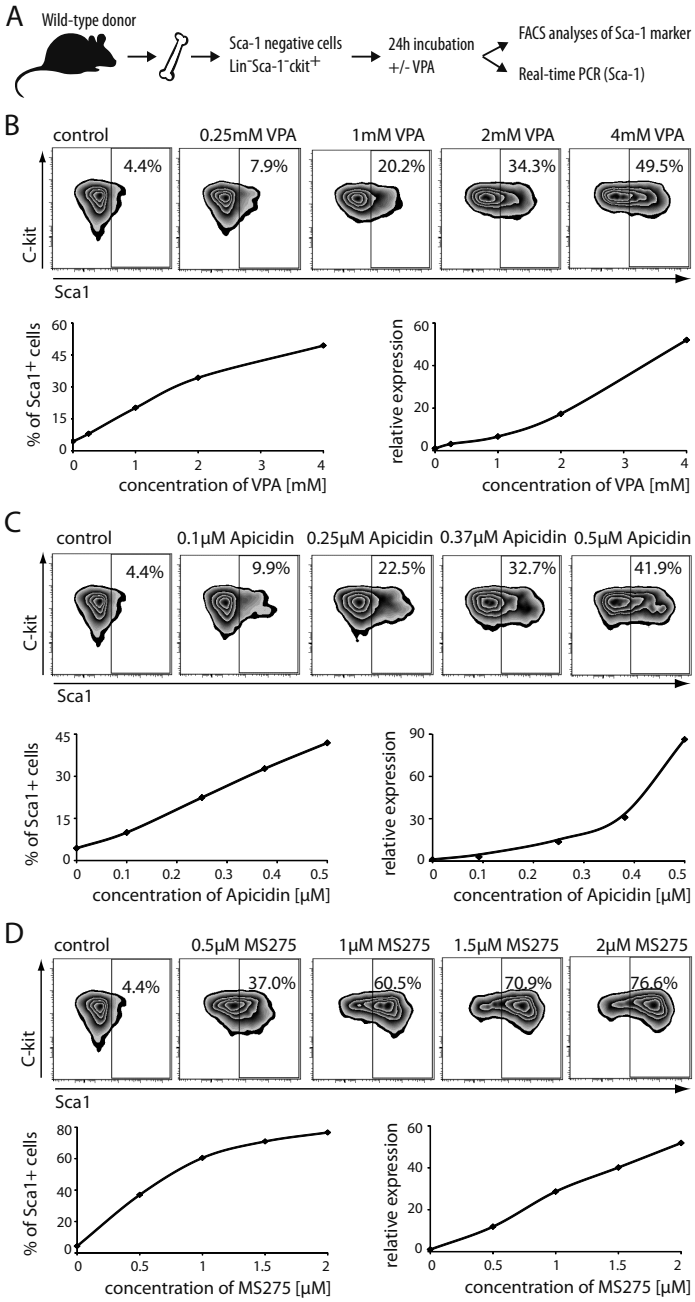

Figure 3. Concentration-dependent Sca-1 induction by distinct HDIs. (A) Experimental design to test effects on distinct HDIs on Sca-1 protein and messenger RNA expression. (B) Short-term (24 hours) effects of increasing concentrations of VPA on Sca-1 expression. (C) Short-term (24 hours) effects of increasing concentrations of Apicidin on Sca-1 expression. (D) Short-term (24 hours) effects of increasing concentrations of MS-275 on Sca-1 expression.

cells (Figure 3D). Additionally, the induction of Sca-1 expression was dependent on continuous presence of HDI because 24 hours after drug removal, 50% of (re-)induced Sca-1^{pos} cells lost the marker expression (Supplementary Figure S1). Our data demonstrate that Sca-1 gene expression can be easily, quickly, and efficiently re-induced on Sca-1^{neg} cells, and that the level of Sca-1 expression can be titrated by varying the HDI concentration.

Sca-1 re-induction correlates with enhanced *in vitro* cell clonogenic activity. Because expression of Sca-1 is of unknown functional relevance, we studied the biological consequences of Sca-1 (re-)induction. Previously, it was shown that VPA treatment can induce self-renewal activity of committed myeloid progenitors, CMPs and GMPs, as measured by methylcellulose replating activity.[18] To test the functionality of cells that regained Sca-1 expression, we used the previously described CFU–granulocyte-macrophage assay setup. Committed myeloid progenitors (CMPs and GMPS) and more differentiated cells, defined by Lin$^+$Sca-1^{neg} phenotype, were FACS-sorted and incubated for 24 hours with or without VPA. After incubation cells were re-sorted and Sca-1^{neg} and Sca-1^{pos} cells were plated into CFU assays (Figure 4A). Because Sca-1 expression and clonogenic activity of hematopoietic cells were shown to be HDI dependent (Supplementary Figure S1), VPA was present during the entire culturing period in case of re-sorted Sca-1^{neg} as well as Sca-1^{pos} cells. Primary colony counts showed enhanced CFU activity of cells that reacquired Sca-1 expression and that were derived from VPA-stimulated CMP as well as VPA-stimulated and non-stimulated Lin$^+$Sca-1^{neg} cells. However, no differences in primary CFU activity between Sca-1^{neg} and Sca-1^{pos} cells could be observed for non-stimulated CMP and both non- and VPA-stimulated GMP (Figure 4B). Strikingly, the self-renewal potential of Sca-1^{pos} and Sca-1^{neg} cell populations as tested by colony replating showed that for all tested cell populations (CMP, GMP, and Lin$^+$Sca-1^{neg}), both VPA-stimulated and non-stimulated, the majority of replating activity was derived from cells with reacquired Sca-1 expression. In addition, VPA-stimulated cells that regained Sca-1 expression showed much higher replating potential compared with non-stimulated Sca-1^{pos} cells (Figure 4C). In addition, a higher number of VPA-stimulated cells regained Sca-1 expression compared with non-VPA-stimulated cells. These data indicate that VPA-enhanced replating capacity of CMP, GMP, and Lin$^+$Sca-1^{neg} cells and more than 90% of the replating capacity resided in cells with regained Sca-1 expression.

Sca-1 is dispensable for VPA-induced enhanced activity of hematopoietic cells. Because our results demonstrate that hematopoietic cells with re-induced Sca-1 expression display enhanced functional activity, we assessed whether Sca-1 is required for the observed effect. To test this, we performed CFU-granulocyte-macrophage assays using cells derived from Sca-1-deficient mice (referred to as Sca-1 KO mice). Committed myeloid cell populations (CMP and GMP) and differentiated cells defined by Lin$^+$ phenotype were FACS-sorted from wild-type and Sca-1 KO mice without Sca-1 marker selection. In this way, CMP, GMP, and Lin$^+$ cells still contained approximately 10%, 2%, and 1% of Sca-1^{pos} cells (determined in wild-type cells (Supplementary Figure S2). Minor differences with data presented on wild-type cells in Figure 4 were observed because cells were not selected on Sca-1 expression at 24 hours and sorting strategies were slightly different. Figure 5A shows that VPA effects on both primary and secondary clonogenic capacity of CMP and GMP were similar between wild-type and Sca-1 KO cells. In addition,

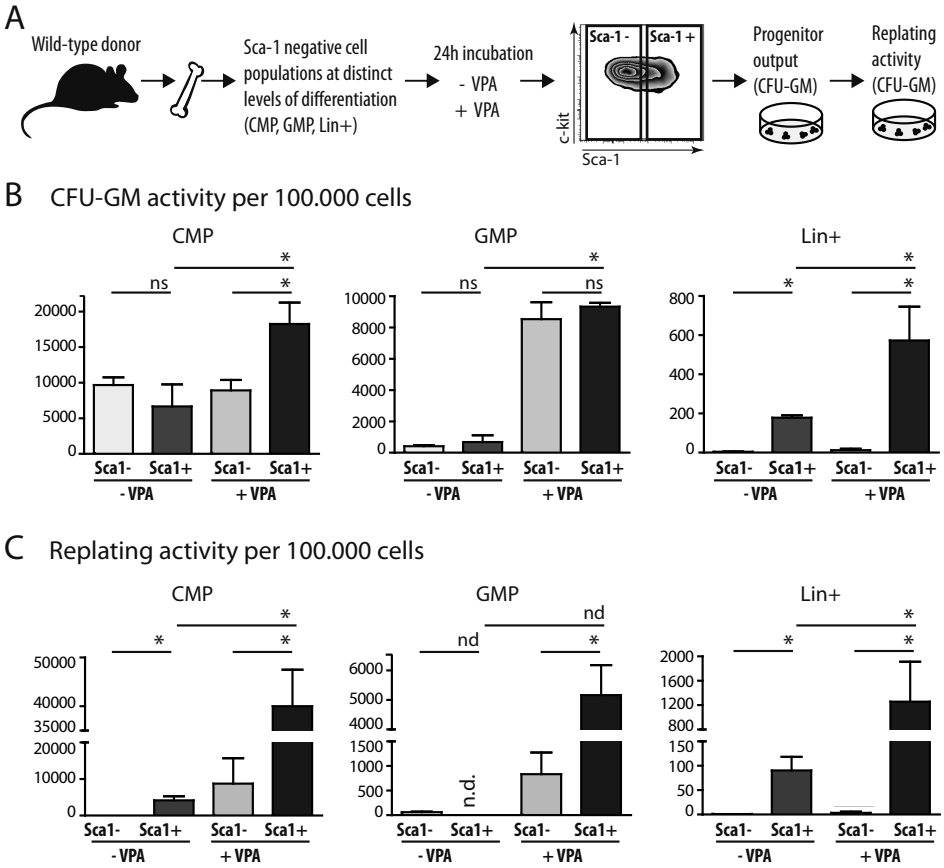

Figure 4. Clonogenic activity of cells with reacquired Sca-1 expression. (A) Experimental design to test functional consequences of reacquired Sca-1 expression on hematopoietic cells that were negative for Sca-1. (B) Primary CFU-granulocyte-macrophage activity of Sca-1^{neg} and Sca-1^{pos} cells derived from VPA-stimulated and VPA-non-stimulated CMP, GMP, or Lin^{+}Sca-1^{neg} cells after 24 hours culture. (C) Clonogenic replating potential of Sca-1^{neg} and Sca-1^{pos} cells derived from VPA-stimulated and VPA-non-stimulated CMP, GMP, or Lin^{+}Sca-1^{neg} cells after 24 hours culture. The replating activity of non-VPA-stimulated GMP could not be determined due to low cell numbers in Sca-1^{pos} samples (only 0.6% of GMP induce Sca-1 without VPA stimulation) and therefore too low colony numbers. The difference between groups was evaluated by Mann-Whitney test using SPSS software; *p <0.05; ND = not determined.

VPA equally enhanced CFU–granulocyte-macrophage capacity of Lin^{+} population of both wild-type and Sca-1 KO cells (Figure 5B). In replating assays, all tested cell populations, from both wild-type and Sca-1 KO mice, showed strongly enhanced self-renewal potential upon VPA exposure (Figure 5C). To exclude the possibility that more primitive cells (Sca-1^{pos}) contribute to the enhanced CFU activity, these results were confirmed in CMP and GMP cell populations that were strongly depleted of Sca-1^{pos} cells (less than 1% Sca-1^{pos} cells) using the SLAM marker profile (CD48^{+} and CD150^{+}) (Supplementary Figure S2). These data clearly demonstrate that the Sca-1 molecule is dispensable for HDI-stimulated enhancement of cell functionality and

replating capacity. Although Sca-1 is not required for the biological effects of VPA, it marks only those cell populations with improved stem/progenitor cell readout upon VPA stimulation.

DISSCUSSION

During the last several years, it has been shown that HDIs, particularly VPA, can retain HSPC activity during *ex vivo* culture.[18-22] However, the molecular events of these effects are largely unknown. In this study, we set out to determine the early molecular response of HSPCs to VPA stimulation. Our genome-wide gene expression studies revealed over-representation of

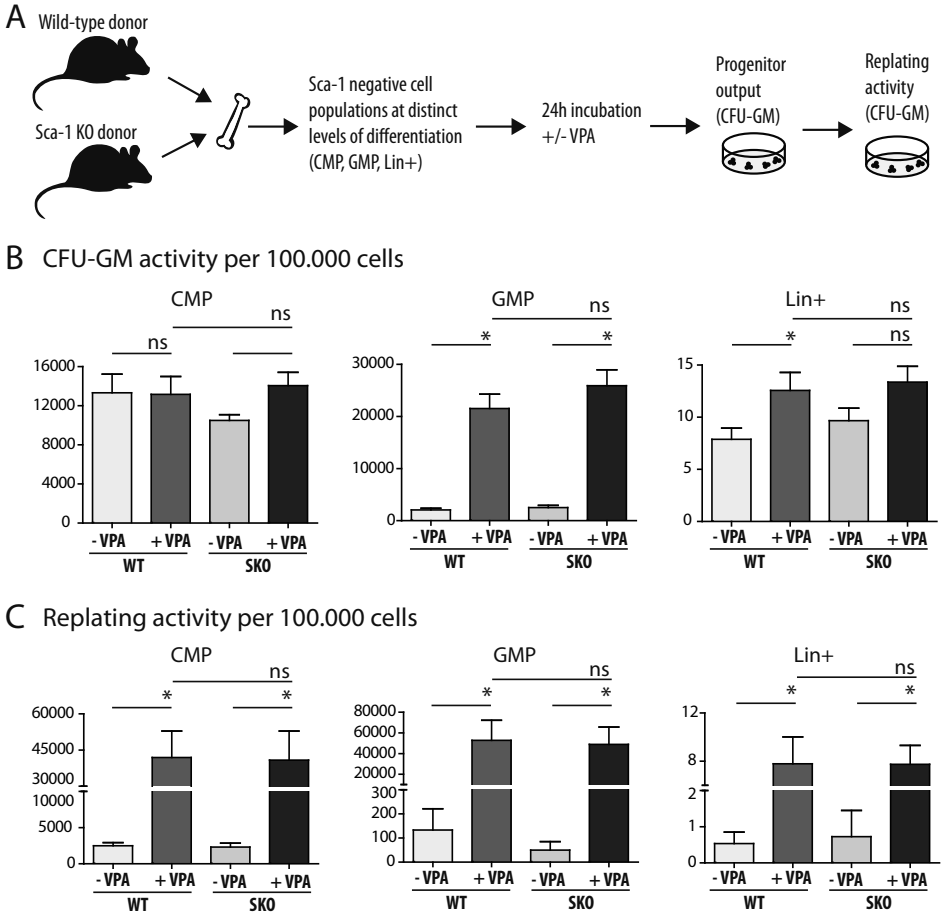

Figure 5. VPA effects on clonogenic activity of Sca-1-deficient hematopoietic cells. (A) Experimental design to test requirement of Sca-1 marker in the VPA-enhanced stem/progenitor cell activity. (B) Primary CFU-GM activity upon VPA stimulation of wild-type and Sca-1 KO-derived CMP, GMP, and Lin$^+$ cells. (C) Clonogenic replating potential upon VPA stimulation of wild-type and Sca-1 KO-derived CMP, GMP, and Lin$^+$ cells. The difference between groups were evaluated by Mann-Whitney test using SPSS software; *p<0.05.

genes with receptor and signal transducer activity and changes in the HSPC surface profile after short (24 hours) VPA treatment. We identified the well-known and widely used HSPC markers Sca-1 and Thy-1 as early VPA targets, and we found that distinct HDIs can readily and effectively induce expression of these markers on HSPCs. Particularly, Sca-1 is included in many murine HSC-sorting strategies, separating the stem cell compartment from a more committed myeloid compartment. Strikingly, after 24 hours culture, VPA not only strongly preserved Sca-1 expression and the LSK phenotype of isolated HSPCs, but it also re-induced Sca-1 on committed progenitors (CMPs and GMPs) that had lost its expression in the process of differentiation, reverting those cells to a more immature LSK phenotype. The reacquired Sca-1 expression was accompanied by increased functional potential, as demonstrated by enhanced self-renewal activity in replating assays compared with Sca-1^{neg} cells. Although Sca-1 was dispensable for observed HDI effects, it serves as a marker of VPA-responsive cells.

HDIs, including VPA, are widely used in anti-cancer therapy, where they lead to cancer cell death. The VPA concentrations used in leukemia treatment seem to have no effect on non-transformed cells, making HDIs well suited for cancer therapy. However, recent studies indicate that VPA displays similar stem cell-enhancing effects on leukemia initiating cells[38,39], suggesting that normal and transformed stem cells can respond uniformly to VPA stimulation and share the same targets. These findings emphasize the importance of understanding the spectrum of both biological and molecular effects of VPA. Our microarray data indicate that, in addition to cell communication and signal/receptor activity, HSPCs respond to VPA also by up-regulation of genes involved in glutathione metabolism. Because glutathione is involved in detoxification and anti-oxidation mechanisms, it can play a role in the selectiveness of anti-cancer drugs, such as HDIs, toward transformed cells.[40,41] Additionally, upregulation of genes involved in chromatin remodeling events, such as *Hmgn3*, *Tshz3*, or *Prkcb1*, were also observed after VPA stimulation, indicating effects on epigenetic mechanisms. The ability of all tested HDIs (VPA, MS-275, and Apicidin) to induce Sca-1 strongly suggest that regulation of Sca-1 gene expression is epigenetically controlled and is due to specific HDI deacetylation inhibitory activity and not off-target effects of VPA. Additionally, because both Apicidin and MS-275 (HDAC class I inhibitors) could effectively induce Sca-1, these suggest that neither inhibition of class II HDACs nor HDAC-1 or -2 seem to be crucial for regulation of Sca-1 gene expression. VPA treatment could therefore affect cell functionality by modulating chromatin conformation at key regulatory genes, which is considered to be important in determining the fate of HSCs.

This study shows that VPA treatment can induce an LSK-like phenotype in committed myeloid progenitors by reactivating Sca-1. The committed cells reacquired replating activity, suggesting partial de-differentiation. Interestingly, addition of VPA to culture conditions used to reprogram differentiated cells into induced pluripotent stem cells has been shown to strongly improve the efficiency of induced pluripotent stem cell production.[41] The acquisition of stem cell properties is accompanied by epigenetic variations, including genome-wide chromatin decondensation.[42] In addition, accessible chromatin is a general feature of stem cells.[3,43] We postulate that VPA, by inhibiting deacetylation events, leads to hyperacetylation and confers a more open chromatin conformation to target cells, which can facilitate the de-differentiation process. Together, these data indicate that HDIs, such as VPA, might play a role in cellular reprogramming. It is likely that

for full reprogramming, additional factors are needed, such as key (HSC) transcription factors, because naturally occurring HSPC de-differentiation has not been reported.

In combination with other markers, Sca-1 is the most commonly used marker to enrich for adult murine HSCs and also other adult stem cell types. In addition, Sca-1 is up-regulated in a variety of mammary tumors, likely reflecting cancer stem cell population, and suggesting its role in cell stemness.[44,45] HSCs deficient for Sca-1 displayed a competitive disadvantage upon serial transplantations, suggesting HSC self-renewal defects.[13,17] In addition, Sca-1 overexpression abrogated myeloid colony formation of mouse and human hematopoietic precursors, suggesting a role for Sca-1 in lineage specification.[13] Sca-1 has also been suggested to play a role in activation of dormant primitive HSC by interferon-α.[46] Multiple attempts have been made to identify a function of Sca-1, but no consensus has been reached and its role in stem cells has yet to be elucidated. Based on its high similarity with other Ly6 superfamily members, Sca-1 was hypothesized to act via receptor-ligand interactions and/or via modification of lipid rafts and subsequently cell signaling.[12;48] However, to date, ligands for Sca-1 have not been found, suggesting that it might not function via classical receptor-ligand binging, but alternatively as a co-regulator of signaling pathways. Here, we report on over-representation of genes implicated in receptor and signal transducer activity, suggesting that VPA-induced Sca-1 expression might influence cell signaling as a coregulator of signaling pathways, for instance, by modulating lipid rafts composition.[12,47]

Collectively, our data demonstrate that VPA can induce important effects on hematopoietic stem and progenitor cell self-renewal and differentiation. It was shown that Sca-1 is an early VPA target and that its expression is efficiently induced by VPA. Finally, we show that Sca-1 can serve as a marker of HDI-responsive cells, reflecting enhanced cell functionality.

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CONFLICT OF INTEREST DISCLOSURE

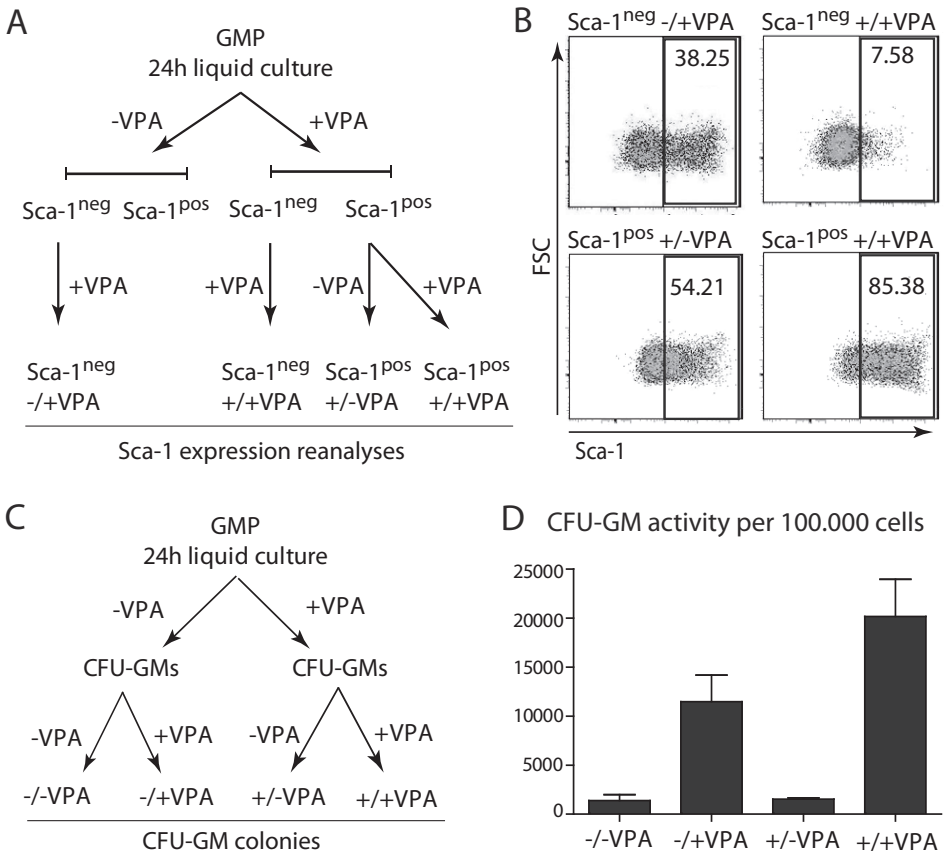
No financial interest/relationships with financial interest relating to the topic of this article have been declared.

REFERENCES

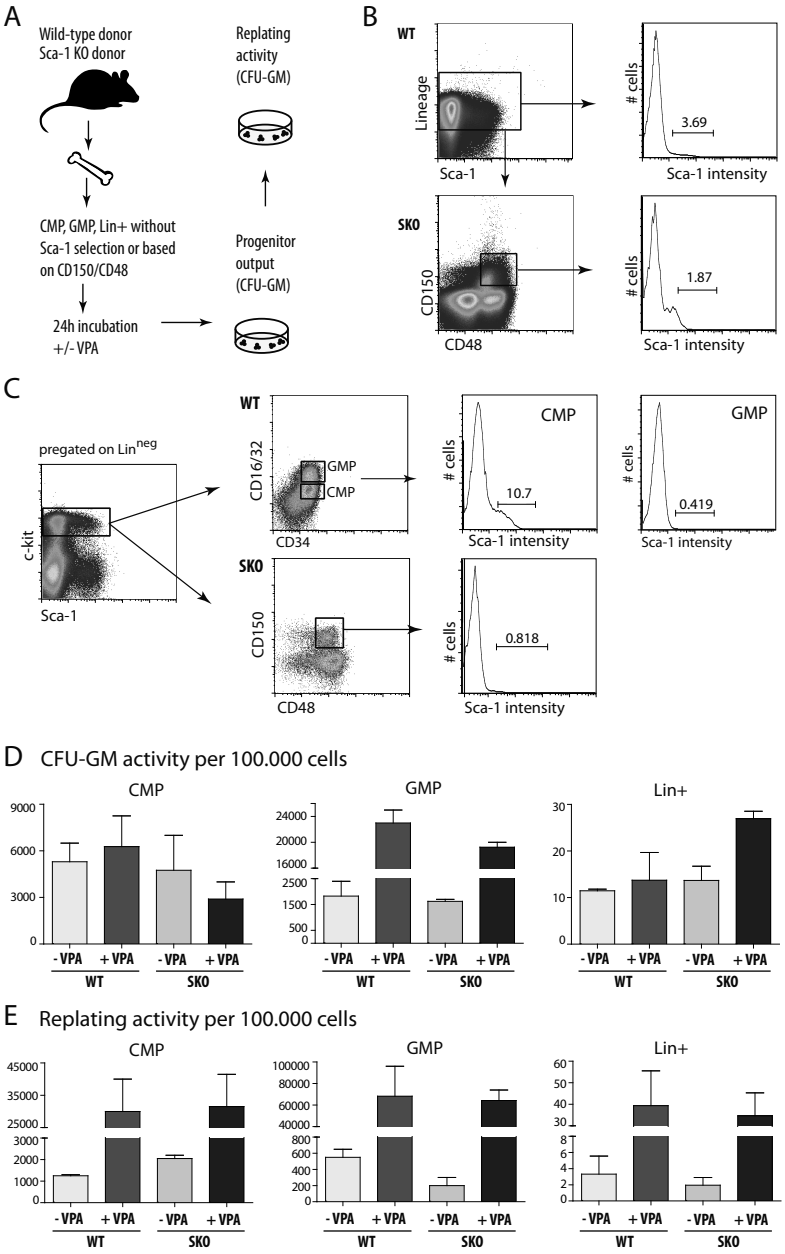
1. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature. 2000;404:193–197.
2. Kondo M, Weissman I, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell. 1997; 91:661–672.
3. Akashi K, He X, Chen J, et al. Transcriptional accessibility for genes of multiple tissues and hematopoietic lineages is hierarchically controlled during early hematopoiesis. Blood. 2003;101:383–389.
4. Passegue E, Jamieson CH, Ailles LE, Weissman IL. Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? Proc Natl Acad Sci U S A. 2003; 100(suppl_1):11842–11849.
5. Adolfsson J, Mansson R, Buza-Vidas N, et al. Identification of Flt3L lympho-myeloid stem cells lacking erythro-megakaryocytic potential: a revised road map for adult blood lineage commitment. Cell. 2005; 121:295–306.
6. M_ansson R, Hultquist A, Luc S, et al. Molecular evidence for hierarchical transcriptional lineage priming in fetal and adult stem cells and multipotent progenitors. Immunity. 2007;26:407–419.
7. Kulessa H, Frampton J, Graf T. GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thromboblasts, and erythroblasts. Genes Dev. 1995;9:1250–1262.
8. Xie H, Ye M, Feng R, Graf T. Stepwise reprogramming of B cells into macrophages. Cell. 2004;117:663–676.
9. Laiosa CV, Stadtfeld M, Xie H, de Andres-Aguayo L, Graf T. Reprogramming of committed T cell progenitors to macrophages and dendritic cells by C/EBPa and PU.1 transcription factors. Immunity. 2006;25:731–744.
10. Challen GA, Boles N, Lin KK, Goodell MA. Mouse hematopoietic stem cell identification and analysis. Cytometry Part A. 2009;75A: 14–24.
11. de Bruijn MFTR, Ma X, Robin C, Ottersbach K, Sanchez M, Dzierzak E. Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. Immunity. 2002;16:673–683.
12. Holmes C, Stanford WL. Concise review: stem cell antigen-1: expression, function, and enigma. Stem Cells. 2007;25:1339–1347.
13. Bradfute SB, Graubert TA, Goodell MA. Roles of Sca-1 in hematopoietic stem/progenitor cell function. Exp Hematol. 2005;33:836–843.
14. Ma X, Robin C, Ottersbach K, Dzierzak E. The Ly-6A (Sca-1) GFP transgene is expressed in all adult mouse hematopoietic stem cells. Stem Cells. 2002;20:514–521.
15. Hanson P, Mathews V, Marrus SH, Graubert TA. Enhanced green fluorescent protein targeted to the Sca-1 (Ly-6A) locus in transgenic mice results in efficient marking of hematopoietic stem cells in vivo. Exp Hematol. 2003;31:159–167.
16. Uchida N, Weissman IL. Searching for hematopoietic stem cells: evidence that Thy-1.1lo Lin-Sca-1+ cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. J Exp Med. 1992;175:175–184.
17. Ito CY, Li CY, Bernstein A, Dick JE, Stanford WL. Hematopoietic stem cell and progenitor defects in Sca-1/Ly-6A-null mice. Blood. 2003;101:517–523.
18. Walasek MA, Bystrykh LV, van den Boom V, et al. The combination of valproic acid and lithium delays hematopoietic stem/progenitor cell differentiation. Blood. 2012;29(119):3050–3059.
19. Bug G, Gul H, Schwarz K, et al. Valproic acid stimulates proliferation and self-renewal of hematopoietic stem cells. Cancer Res. 2005;65: 2537–2541.
20. DeFelice L, Tatarelli C, Mascolo MG, et al. Histone deacetylase inhibitor valproic acid enhances the cytokine-induced expansion of human hematopoietic stem cells. Cancer Res. 2005;65:1505–1513.
21. Vulcano F, Milazzo L, Ciccarelli C, et al. Valproic acid affects the engraftment of TPO-expanded cord blood cells in NOD/SCID mice. Exp Cell Res. 2012;318:400–407.
22. Milhem M, Mahmud N, Lavelle D, et al. Modification of hematopoietic stem cell fate by 5aza 2′-deoxycytidine and trichostatin A. Blood. 2004;103:4102–4110.
23. Zini R, Norfo R, Ferrari F, et al. Valproic acid triggers erythro/megakaryocyte lineage decision through induction of GFI1B and MLLT3 expression. pii: S0301–472X(12)00344-X. Exp Hematol. 2012 Aug 10; http://dx.doi.org/10.1016/j.exphem.2012.08.003. [Epub ahead of print].
24. Chow A, Lucas D, Hidalgo A, et al. Bone marrow CD169þ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. J Exp Med. 2011;208: 261–271.
25. Christopher MJ, Rao M, Liu F, Woloszynek JR, Link DC. Expression of the G-CSF receptor in monocytic cells is sufficient to mediate hematopoietic progenitor mobilization by G-CSF in mice. J Exp Med. 2011;208:251–260.
26. Winkler IG, Barbier V, Wadley R, Zannettino AC, Williams S, Levesque JP. Positioning of bone marrow hematopoietic and stromal cells relative to blood flow in vivo: serially reconstituting hematopoietic stem cells reside in distinct nonperfused niches. Blood. 2010;116: 375–385.
27. Altman D. Practical Statistics for Medical Research. London: Chapman and Hall Books; 1991.
28. Pukelsheim F. The three sigma rule. Am Stat. 1994;48:88–91. 29. Backes C, Keller A, Kuentzer J, et al. GeneTraildadvanced gene set enrichment analysis. Nucleic Acids Res. 2007;35:W186–W192.
29. van Os RP, Dethmers-Ausema B, de Haan G. In vitro assays for cobblestone area-forming cells, LTC-IC, and CFU-C. Methods Mol Biol. 2008;430:143–157.
30. Van Lint C, Emiliani S, Verdin E. The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. Gene Exp. 1996;5:243–253.
31. Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG, Davidsen SK. Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. Mol Cancer Ther. 2003;2:151–163.

32. Mitsiades CS, Mitsiades NS, McMullan CJ, et al. Transcriptional signature of histone deacetylase inhibition in multiple myeloma: biological and clinical implications. Proc Natl Acad Sci U S A. 2004;101: 540–545.
33. Min IM, Pietramaggiori G, Kim FS, Passegue E, Stevenson KE, Wagers AJ. The transcription factor EGR1 controls both the proliferation and localization of hematopoietic stem cells. Cell Stem Cell. 2008;2:380–391.
34. Tang W, Borel AG, Abbott FS. Conjugation of glutathione with a toxic metabolite of valproic acid, (E)-2-propyl-2,4-pentadienoic acid, catalyzed by rat hepatic glutathione-S-transferases. Drug Metab Dispos. 1996;24:436–446.
35. Kiang TKL, Teng XW, Surendradoss J, Karagiozov S, Abbott FS, Chang TKH. Glutathione depletion by valproic acid in sandwichcultured rat hepatocytes: role of biotransformation and temporal relationship with onset of toxicity. Toxicol Appl Pharmacol. 2011;252: 318–324.
36. Khan N, Jeffers M, Kumar S, et al. Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors. Biochem J. 2008;409:581–589.
37. Bug G, Schwarz K, Schoch C, et al. Effect of histone deacetylase inhibitor valproic acid on progenitor cells of acute myeloid leukemia. Haematologica. 2007;92:542–545.
38. Leiva M, Moretti S, Soilihi H, et al. Valproic acid induces differentiation and transient tumor regression, but spares leukemia-initiating activity in mouse models of APL. Leukemia. 2012;26:1630–1637.
39. Ungerstedt JS, Sowa Y, Xu WS, et al. Role of thioredoxin in the response of normal and transformed cells to histone deacetylase inhibitors. Proc Natl Acad Sci U S A. 2005;102:673–678.
40. Huangfu D, Maehr R, Guo W, et al. Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. Nat Biotechnol. 2008;26:795–797.
41. Grafi G. The complexity of cellular dedifferentiation: implications for regenerative medicine. Trends Biotechnol. 2009;27:329–332.
42. Gaspar-Maia A, Alajem A, Meshorer E, Ramalho-Santos M. Open chromatin in pluripotency and reprogramming. Nat Rev Mol Cell Biol. 2011;12:36–47.
43. Kruger JA, Kaplan CD, Luo Y, et al. Characterization of stem cell–like cancer cells in immune-competent mice. Blood. 2006;108:3906–3912.
44. Mulholland DJ, Xin L, Morim A, Lawson D,Witte O,Wu H. Lin–Sca- 1+CD49fhigh stem/progenitors are tumor-initiating cells in the Ptennull prostate cancer model. Cancer Res. 2009;69:8555–8562.
45. Essers MAG, Offner S, Blanco-Bose WE, et al. IFNalpha activates dormant haematopoietic stem cells in vivo. Nature. 2009;458:904– 908.
46. van de Rijn M, Heimfeld S, Spangrude GJ, Weissman IL. Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. Proc Natl Acad Sci. 1989;86:4634–4638.

SUPPLEMENTAL INFORMATION



Supplementary Figure S1. Dependence of Sca-1 expression and clonogenic activity on continuous presence of HDI. (A) Experimental design to test dependence of Sca-1 expression on the presence of VPA. (B) Sca-1 expression re-analyses of VPA treated and untreated cells. Cells were pre-gated based on SSC, FSC and propidium ionide for cell viability. (C) Experimental design to test dependence of clonogenic activity on the presence of VPA. (D) Primary CFU-GM activity of pre-treated or untreated hematopoietic cells.



Supplementary Figure S2. Sca-1 expression and clonogenic activity of cells tested for increased functionality in WT and Sca-1 deficient hematopoietic cells. (A) Experimental design to test requirement of Sca-1 marker in the VPA-enhanced stem/progenitor cell activity. (B) Sorting strategies of hematopoietic populations from WT and Sca-1 deficient mice and Sca-1 percentages within sorted cell populations. In Lin^+ cells only 4% of cells express Sca-1 in WT cells. Among $Lin^+CD150^+CD48^+$ cells less than 2% of cells express Sca-1. (C) Cells sorted as CMP and GMP were pre-gated based on SSC, FSC and propidium ionide for cell viability, and lineage negative cells. When Sca-1 expression was ignored, CMP and GMP still contained about ►

Supplemental information available online on request

Table S1. Gene expression dataset on 24h cultured cells with or without VPA.

Table S2. Gene Ontology classification of identified VPA-early response targets in hematopoietic cells.

▶ 10% and less than 1% Sca-1^{pos} cells, respectively. Within Lin$^-$c-kit$^+$CD150$^+$ CD48$^+$ cells less than 1% of cells express Sca-1. (D) Primary CFU-GM activity upon VPA stimulation of wild type and Sca-1 KO derived CMP (Lin$^-$c-kit$^+$CD150$^+$CD48$^+$CD16/32^{mid}CD34$^+$), GMP (Lin$^-$c-kit$^+$CD150$^+$CD48$^+$CD16/32^{high}) and Lin$^+$CD150$^+$CD48$^+$ cells where Sca-1 expression was ignored but CD150 and CD48 were included as progenitor cell markers. (E) Clonogenic re-plating potential upon VPA stimulation of wild type and Sca-1 KO derived CMP, GMP and Lin$^+$ cells where Sca-1 expression was ignored but CD150 and CD48 were included as progenitor cell markers.

CHAPTER 6

SUMMARIZING DISSCUSION & FUTURE PERSPECTIVES

SUMMARY

Expansion of fully functional self-renewing, multipotent and engraftable HSCs is expected to be highly beneficial for stem cell-based therapies and represent a long-standing goal of stem cell biology. Despite intensive studies, defining the *in vitro* culture conditions that can maintain HSC characteristic properties of self-renewal and lack of differentiation has proven to be difficult. Therefore there has been much interest in understanding molecular (intrinsic) and environmental (extrinsic) determinants governing HSC fate decisions, as well as manipulation of HSC culture conditions to efficiently expand HSCs *ex vivo*. In this thesis we studied the biological and molecular effects of distinct, both intrinsic and extrinsic factors on HSC characteristics, particularly on stem cell maintenance, expansion and differentiation.

Chapter 2 provides an introduction to the concept and goals of HSCs expansion studies. We reviewed here past and recent HSC expansion approaches, briefly summarizing molecular factors playing a role in HSC fate decisions, further focusing on environmental cues, such as cytokines, developmental factors and small molecules, to regulate HSC self-renewal and differentiation in culture. We concluded that the most comprehensive approach to develop optimal HSC expansion conditions may involve a combination of currently studied methods. It is likely that these conditions would involve multiple biological and chemical compounds, which act in concert to induce cell survival and division while simultaneously preventing stem cell differentiation. Finally, we discussed in this chapter the remaining challenges of the field that need to be answered before unlimited *ex vivo* HSC expansion could be achieved.

One of these remaining challenges that could substantially contribute to the success of *ex vivo* HSCs expansion protocols is a better understanding of the molecular mechanisms regulating HSC self-renewal and differentiation. Several approaches can be used to identify intrinsic factors that play a role in HSC fate determination, such as identification of key stem cell specific genetic factors and/or studying the cues underlying known mouse-strain dependent variation in HSC frequencies. By applying these approaches in **chapter 3** we determined whether changes and variation in microRNA expression could influence HSC fate. We performed genome-wide microRNA expression profiling of four developmentally related hematopoietic cell types isolated from the C57BL/6 (B6) and DBA/2 (D2) mice, and we determined the expression dynamics of microRNAs during hematopoietic differentiation. We focused on those microRNAs which expressions were both, down-regulated during stem cell differentiation and that were higher expressed in D2 cells compared to B6 cells, and thus correlated with HSPC frequency. This led to the identification of an evolutionary conserved microRNA cluster consisting of miR-99b, let-7e and miR-125a. A single member of this miR-cluster, miR-125a was identified to be responsible for the majority of the miR-cluster 99b/let-7e/125a overexpression phenotypes. Overexpression of this miR-cluster, or only miR-125a, in HSPCs increased the *in vitro* stem cell functionality measured by replating and CAFC assays, and conferred apparent competitive advantage in transplantation settings. However, detailed analysis of stem cell compartment revealed that HSCs overexpressing miR-cluster 99b/let-7e/125a were significantly compromised, resulting in stem cell exhaustion. Moreover, we found that mice reconstituted with cells overexpressing the miR-cluster exhibited increased myeloid differentiation, leading to development of myeloproliferative neoplasm that occasionally progressed to leukemia. Finally,

we identified candidate functional targets through which miR-125a could modulate HSPC fate. Results discussed in this chapter clearly show that microRNAs could influence HSC self-renewal and differentiation properties. However, overexpression of miR-125a, identified as a stem cell specific microRNA, after an initial boost of myeloid output cells, negatively influenced primitive HSC numbers, and subsequently exerted a disease phenotype. Therefore, miR-125a does not qualify as a potential candidate for HSCs expansion protocols.

Introduction of genetic material is undesired in clinical HSC expansion protocols as it might result in undesired clinical outcomes. Alternatively, the HSC stemness could be stimulated extrinsically by addition of distinct factors to the HSC cultures. Such conditions should induce cell proliferation in order to increase cell numbers with concomitant stimulation of self-renewal and inhibition of differentiation in order to maintain HSCs characteristics. **Chapter 4** describes the potential of two small chemical molecules, lithium (Li, a simple cation) and valproic acid (VPA, a well-known histone deacetylase inhibitor) to promote maintenance of HSPC in culture by inhibiting cell differentiation. To assess potential anti-differentiation properties of these two compounds, we stimulated HSPC into differentiation with SCF and GM-CSF. We showed that supplementing the HSPC cytokine cultures with the combination of Li and VPA resulted in the preservation of an immature cell phenotype and *in vitro* stem cell functionality, as measured by CFU and CAFC assays. Moreover, anti-differentiation effects of VPA and Li were observed also at the level of committed progenitors, where these compounds re-activated replating activity of CMP and GMP cells. Strikingly, after a 7-day differentiation culture cells exposed to the combination of Li and VPA provided radioprotection to lethally irradiated recipients, shortened the time required for platelet and red blood cell recovery, and enhanced *in vivo* repopulation potential. Anti-differentiation effects of the combination of Li and VPA were confirmed at the molecular level. We showed that VPA and Li synergistically preserved expression of stem cell-related genes and repressed genes involved in differentiation. Additionally, we identified genetic networks and putative targets that are transcriptionally affected by both compounds. This study provides evidence that HSC self-renewal and differentiation can be manipulated chemically by small molecules, representing relatively new approach in expansion studies. Additionally, our data suggest that combinatorial screening of chemical compounds, and other potential self-renewal factors may uncover possible additive/synergistic effects to modulate HSPC self-renewal and differentiation and thereby may lead to novel approaches in *ex vivo* stem cells culturing systems.

Findings that the fate of HSPC can be modulated chemically by the addition of small molecules to the culture media, such as lithium and valproic acid (described in chapter 4), is promising in light of *ex vivo* HSC expansion. However, at present our understanding of the molecular events of chemical modulation of HSCs is limited. VPA has been used in leukemia treatment as a differentiation therapy. Therefore the identification of gene targets of compounds such as VPA is important to understand the full potential of these molecules. In **Chapter 5** we determined the early molecular response of HSPCs to VPA treatment. Our analysis revealed that overrepresentation of genes involved in glutathione metabolism, receptor and signal transducer activity and changes in the HSPCs surface profile following short, 24h VPA treatment. We identified the well-known and widely used HSPC markers Sca-1 and Thy-1 as early VPA targets, and we found that distinct HDIs can readily and effectively induce expression of these markers

on HSPCs. Strikingly, after 24h culture, VPA not only strongly preserved Sca-1 expression and the LSK phenotype of isolated HSPCs, but it also re-induced Sca-1 on committed progenitors (CMPs and GMPs) that had lost its expression in the process of differentiation, reverting those cells to a more immature LSK phenotype. Moreover, Sca-1 re-induction coincided with induced self-renewal capacity as measured by *in vitro* replating assays, while Sca-1 itself was dispensable for the functional effects of VPA as demonstrated using Sca-1 deficient progenitor cells. These data provide the first analysis of direct VPA targets in primitive hematopoietic cells and show that Sca-1 is a faithful marker to identify HDI-responsive cells after *in vitro* culture.

FUTURE PERSPECTIVE

Expansion of HSCs has been a scientific struggle for many years and despite extensive research scientists have had difficulties trying to amplify or even maintain hematopoietic stem cells in culture. Major roadblocks have remained unanswered. What puts or keeps HSCs in a self-renewal division mode? What are the golden standard culture conditions to support unlimited HSC self-renewal and proliferation? Finally, to what extent could it be possible to generate fully functional HSCs, and their derivatives *ex vivo*? Limited understanding how HSC fate decisions are controlled *in vivo* translate into limited success rate of *ex vivo* HSC expansion protocols. Therefore, to be able to effectively manipulate the hematopoietic system we first have to understand it. Identification of signals controlling HSC self-renewal and differentiation *in vivo*, such as intrinsic factors as well as signals from the HSC microenvironment (soluble growth factors, extracellular matrix and stroma components), and understanding how are they correlated with each other seems to be crucial for the understanding of HSC fate regulation and the success of HSC expansion. In this thesis we examined whether and how recently discovered new intrinsic molecules, microRNAs, can affect HSC self-renewal and differentiation. Such fundamental studies are important as they add missing pieces to the puzzle of HSC regulation and might eventually lead to the discovery of new molecules that could be used to expand HSCs *in vitro*. Developments of genome-wide state-of-the-art tools to study intrinsic self-renewal determinants, such as mRNA expression, microRNA expression, protein expression, post-transcriptional modifications, transcription factor binging sites and epigenetic marks, offer the possibility of better insights into mechanisms controlling HSC self-renewal. However, the limiting number of HSC per mouse makes most of these types of analysis challenging for the hematopoietic system that have yet to be overcome. One may think that technological progress and identification of new levels of gene regulation would make it easier to identify stem cell factors, but key determinants regulating cell stemness at present remain unknown. Additionally, research focused on single determinants, such as mRNA or microRNA only, do not fully reflect complexity of the self-renewal mechanism. Explanation and understanding of complex phenomena, such as self-renewal, might require an integrative approach. Likely not only one gene is responsible for the stem cell phenotype and more factors should be considered as a read-out for stem cell activity. Constructing regulatory networks (as in chapter 4) would help to oversee which gene network or related genes are involved in co-regulation of important biological processes. The ultimate goal however, would be to construct regulatory

networks controlling HSC fate decisions that integrate multiple distinct determinants, such as mRNAs, non-coding RNAs or epigenetic factors, and which eventually will allow us to predict, model and potentially control behavior of HSCs in culture.

Difficulties in *ex vivo* expansion seem not to be shared by some of the other tissue-specific stem cell systems. Maintenance or expansion of stem cells from tissues such as embryo, epidermis, intestinal epithelia or retina, could be relatively easily achieved by culturing with a defined mix of factors that keeps the cells in a self-renewal mode and prevents successive differentiation. There could be several possible reasons for this difference, such as the stringency of available stem cell assays, growth factor dependence or stroma involvement. A hypothesized universal self-renewal signature was an interesting concept that could help to translate the achievement from other stem cell tissues to HSC. However, studies comparing mRNA expression profiles of HSC, embryonic and neural stem cells led to disappointing results, showing very little or no overlap between tested stem cell systems, suggesting that such a thing as acommon self-renewal signature most likely does not exists. Unfortunately, the reason for those differences in expansion feasibility between stem cells from distinct tissues is unknown yet.

It has been speculated that the answer to successful HSC expansion could also be found by defining the cellular composition and complex molecular signals in the AGM region during HSC development. Several developmental factors, such as Wnt and Notch, have been shown to help to maintain adult HSC in culture. However, this concept is a matter of onging discussion. A possible alternative to improve HSC expansion protocols, not based on prior knowledge, is "blind" screening of hundreds of thousands of distinct factors for potential self-renewal enhancing effects. The first reports using high-throughput screening of chemical compound libraries demonstrate utility of this approach, and led to the discovery of compounds, such as PGE_2 or SR1 (an aryl hydrocarbon receptor antagonist), representing new promising HSC expansion factors. Additionally, it has been show that full replacement of HSC cytokines could be beneficial for *ex vivo* HSC expansion by modulating strength of the signal induced by the cytokines. A small molecule agonist of c-Mpl receptor (receptor for a well-know and widely used HSC cytokine, Tpo) has been shown to improve HSC maintenance in culture compared with Tpo. Nowadays, thousands of molecules are screened for their effects on HSC fates and the utility of these factors for *in vitro* stem cell expansion is being investigated. The effect and stability of small molecules might be easier to control, and therefore it is possible that such compounds might replace hematopoietic factors in future expansion protocols. In this thesis we tested the ability of two small chemical molecules, lithium and valproic acid (chapter 4 and 5), as a new alternative to control HSC behavior extrinsically during culture. The ability to manipulate *in vitro* HSC properties chemically by small compounds opened a new avenue of possibilities for *ex vivo* HSC expansion protocols and also offers future possibility to design small molecules that would specifically and efficiently target key intrinsic HSC self-renewal factors.

Ex vivo modeling of the BM niche is a new approach aimed to improve *in vitro* HSC expansion protocols and is based on the concept of the importance of the *in vivo* HSC niche for their maintenance. *In vitro* cultures of HSC can be stroma-free or stroma-dependent. Although stroma-free cultures are easier to standardize and maintain, it has been suggested that stroma-dependent cultures provide a more physiological environment that could be beneficial for *in vitro* HSC expansion cultures. However, the disadvantage of such an approach is the introduction of

foreign human or animal antigens to the HSC expansion product. Here BM engineering offers an alternative solution. By the use of the naturally occurring peptides or synthetic polymers it tries to mimic the three-dimensional (3D) BM HSC niche. The idea of these biomaterial-based 3D-like matrix cultures is to provide structural support and opportunity for cell migration and adhesion that occurs in physiological conditions. Up till now, only a few systems for HSC culture using biomaterials have been proposed leaving the field of BM modeling wide-open. Also here, the better knowledge how exactly stem cells are regulated could facilitate the definition of key contact vs non-contact stem cell factors, thereby resulting in the most optimal and fully defined culture conditions. Furthermore, once defined and efficient HSC expansion protocols are established, the use of continuous perfusion culture systems or bioreactors could allow for the development of a therapy-suitable HSC product by providing controlled culture conditions, optimization, standardization and scale-up, making the end product predictable and free of contaminants.

When discussing the future perspectives of HSC expansion studies the recent discovery of induced pluripotent stem cells (iPSCs) needs to be mentioned. Studies by the Yamanaka lab showed that somatic cells could be reprogrammed and de-differentiated into stem cell-like stage by introduction of (in this case) four key embryonic stem cells transcription factors. Such iPSC cells could be then further differentiated into HSC. Alternatively differentiated hematopoietic cells or any other somatic cells that could be easily harvested from the patient's might be possibly directly reprogrammed into HSC. This discovery can potentially revolutionize the field of HSC expansion and offers the possibility of production of unlimited numbers of stem cells from patients own somatic cells. However, there are many questions to be addressed before cellular reprogramming could become a real alternative to traditional HSC expansion approaches.

The biological properties and clinical potential of HSCs elicit continued scientific, commercial and public interest. Our ability to expand HSC *ex vivo* could benefit stem cell transplantation and gene therapy protocols. In recent years, combined efforts of the scientific and industrial community to improve HSC expansion have been promising. Most of the recent developments, such as screening of molecule libraries, developing 3D scaffolds mimicking the BM niche, or use of bioreactors, are the result of such collaborations. Scientifically-driven approaches for new discoveries combined with systematic industrial-based knowledge and experience might in the future offer viable HSC expansion protocols. Although HSCs are the best studied stem cell system, at present the secret of their self-renewal remains hidden, and so does our ability to manipulate HSC *in vitro*. With many remaining questions it is realistic to say that we are just beginning to understand how HSCs properties are regulated and how to use this knowledge to manipulate culture conditions of HSC for clinical purposes. Additionally, development of reliable and good *in vivo* models for human stem cell read-out would help to better validate existing and future stem cell protocols. Based on HSC behavior and their expansion potential *in vivo,* we believe that unlimited *in vitro* HSC amplification is feasible. However with only small steps behind us, achieving this "holy grail" of expansion research might require additional years of research. What the future of *ex vivo* HSC expansion will bring is an intriguing question. What would be the winning combination of HSC expansion factors for clinical *ex vivo* HSC protocols? Based on present knowledge it is feasible to say that it would be a combination of several factors, such as cytokines and small molecules, and just the right ratio to induce the perfect balance between proliferation and self-renewal.

APPENDICES

Summary in Dutch
Summary in English
Summary in Polish
Acknowledgements
Curriculum Vitae

NEDERLANDSE SAMENVATTING (VOOR NIET-INGEWIJDEN)

Hematopoiese is het proces van bloedcelontwikkeling en de uiteindelijke productie van rijpe bloedcellen. Bloed bestaat uit vele verschillende, gespecialiseerde cellen die een belangrijke rol spelen in een organisme. Ze vervullen uiteenlopende functies zoals vervoer van zuurstof naar alle weefsels in het lichaam (rode bloedcellen), verdediging tegen pathogenen (witte bloedcellen) en bloedstolling (bloedplaatjes). Veel van deze gespecialiseerde, rijpe cellen hebben een zeer korte levensduur en ze moeten dus voortdurend worden vervangen. Ongeveer 10^{11}–10^{12} nieuwe bloedcellen worden hiervoor dagelijks geproduceerd. Deze productie van nieuwe cellen is afhankelijk van een kleine populatie van bloedvormende cellen, namelijk hematopoïetische stamcellen (HSC). HSC kunnen zich differentiëren in elk type van volwassen bloedcellen, maar ze kunnen ook hun eigen populatie in stand houden in een proces dat zelfvernieuwing (self-renewal) wordt genoemd. De balans tussen deze twee processen is cruciaal voor een organisme want het maakt een snelle en robuuste reactie op fysiologische stress, zoals bloedverlies, infectie en verwonding (multilineaïre differentiatie) mogelijk en bloed productie is zo gegarandeerd voor een leven lang (zelfvernieuwing).

De eigenschappen van HSCs maken deze cellen een aantrekkelijke bron voor een breed scala van op stamcellen gebaseerde therapieën waar herstel van bloedcellen of van normale hematopoïese nodig is. Dit is onder andere het geval bij de behandeling van kanker, beenmergfalen, immuundeficiënties en genetische aandoeningen, waarbij HSC transplantatie een levensreddende procedure kan zijn. Echter, ieder mens is anders en om een transplantatie succesvol te maken, moeten de HSC van donor en de patiënt immunologisch worden afgestemd. Een goede match voorkomt afstoting van de getransplanteerde cellen en immuunreacties van deze cellen die gericht zijn tegen de ontvanger. De beperkte beschikbaarheid van geschikte HSC uit beenmerg en gemobiliseerde perifere bloed beperkt het gebruik ervan. Het gebruik van navelstrengbloed (UCB) als bron van stamcellen is eerder geschikt, doordat een kleine mismatch mogelijk is. Het aantal stamcellen in een UCB is echter niet voldoende voor transplantatie in een volwassene, wat ook het gebruik van UCB beperkt. Daarom wordt veel onderzoek gedaan naar vermeerdering van HSC aantallen (expansie) *ex vivo*, voorafgaand aan de transplantatie. Tegenwoordig kunnen we slechts één patiënt behandelen met de HSC uit een UCB en de uitdaging is om ongedifferentieerde HSC te vermeerderen (via stimulatie van zelfvernieuwing) zodat de aantallen voldoende zijn voor de behandeling van volwassen patiënten of zelfs meerdere patiënten. Het succes van *ex vivo* stamcel expansie protocollen is afhankelijk van ons vermogen om het gedrag van HSC te manipuleren; om de celdeling te stimuleren en het gelijktijdig behouden van de cel in een ongedifferentieerde staat. De ontwikkeling van dergelijke protocollen vereist het begrijpen van de mechanismen die HSC zelfvernieuwing en differentiatie beïnvloeden, en uiteindelijk het definiëren van de belangrijkste factoren die HSC zelfvernieuwingsceldelingen stimuleren. In dit proefschrift hebben we de mogelijkheden van verschillende factoren om HSC zelfvernieuwing en differentiatie te reguleren onderzocht met het uiteindelijke doel mogelijkheden voor *ex vivo* stamcellen expansie te verkennen. Tijdens de celdeling moet de HSC kiezen tussen zelfvernieuwing en differentiatie, en de balans tussen deze twee keuzes bepaalt de uitkomst van *ex vivo* expansie cultuur. Het lot van HSC wordt gereguleerd door signalen zowel van binnenin de cel (intrinsiek) als signalen van

buitenaf (extrinsiek). In hoofdstuk 3 hebben we geprobeerd om nieuwe intrinsieke regulatoren van hematopoïese te identificeren. Het lot van de cel is geprogrammeerd in het DNA, een genetische code van de cel die wordt vertaald naar RNA niveau en verder naar eiwitniveau. De genetische code van de cel kan worden beïnvloed door andere intrinsieke moleculen, zoals niet-coderende RNA's, waaronder microRNA's. MicroRNA's zijn korte RNA moleculen die kunnen binden aan RNA en het RNA waaraan zij binden inactiveren zodat dit partner RNA niet functioneel meer is. In hoofdstuk 3 hebben we onderzocht of microRNA's de zelf-vernieuwing van HSC kunnen beïnvloeden en het aantal HSC kunnen vermeerderen. Om dit te onderzoeken, hebben we microRNA expressiepatronen onderzocht tijdens differentiatie in vier verschillende hematopoïetische celtypes (stamcellen, voorlopercellen, en rijpe myeloïde en erythroïde cellen) en uit twee verschillende muizenstammen die verschillen in aantallen HSC's. We hebben ons gericht op die microRNA's die ten eerste actief waren in stamcellen en/of voorlopercellen en minder of nauwelijks actief in rijpe bloedcellen, en ten tweede de microRNA's die het meest actief waren in de muizenstam met de meeste bloedvormende stamcellen. Deze criteria zouden microRNA's kunnen identificeren die betrokken zijn bij de regulatie van stamcellen. Dit leidde tot de identificatie van een evolutionair geconserveerd microRNA cluster bestaande uit miR-99b, let-7e en miR-125a. De functie van dit microRNA cluster werd onderzocht door overexpressie van het hele cluster en individuele leden van het cluster in de beenmergcellen. Hiermee hebben we het effect van de overexpressie in verschillende stamcellen en voorlopercellen getest *in vitro* en *in vivo* assays. We vonden dat cellen na overexpressie van het microRNA 99b/let7e/125a cluster aanvankelijk in een stamcelachtige staat bleven. Maar normale bloedvorming werd verstoord in de muizen die getransplanteerd waren met de miR-cluster cellen. Gedetailleerde analyse van het stamcel compartiment van deze muizen liet zien dat HSC na overexpressie van het miR-cluster 99b/let-7e/125a sterk waren aangetast, waardoor dit na verloop van tijd resulteerde in uitputting van stamcellen. Het miR-125a werd geïdentificeerd als het microRNA in dit cluster dat verantwoordelijk is voor het merendeel van de miR-cluster 99b/let-7e/125a overexpressie fenotypes. Ook zijn in deze studie een aantal kandidaat-partner RNA-moleculen van de geteste microRNAs gevonden. De microRNA 125a kan binden aan deze partners en hun translatie tot eiwit-niveau blokkeren, wat mogelijk het lot van de stamcellen kan beïnvloeden.

Omdat het inbrengen van genetisch materiaal in cellen kan leiden tot ongewenste klinische resultaten na transplantatie (bijvoorbeeld de ontwikkeling van leukemie), zal stimulering van HSC amplificatie door extrinsieke factoren het geprefereerde middel zijn om klinische *ex vivo* stamcellen te expanderen. Echter, tot nu toe is er beperkt succes bij het voorkomen van HSC differentiatie met extrinsieke factoren en zonder genetische manipulatie. Eén van de belangrijkste problemen van *ex vivo* expansie protocollen is het onvermogen om stamceldifferentiatie te voorkomen en gelijktijdig celproliferatie te stimuleren. Recent is aangetoond dat kleine chemische moleculen kunnen worden gebruikt als nieuwe instrumenten voor het manipuleren van het lot van de stamcel. Hoofdstuk 4 beschrijft de mogelijkheden van twee van deze kleine chemische moleculen, namelijk lithium (Li, een eenvoudige kation) en valproïnezuur (VPA, een bekende histon-deacetylase-remmer). Er is onderzocht wat hun effect is op het remmen van stamceldifferentiatie en dus het behoud van HSC in kweek. De toevoeging van deze beide verbindingen, Li en VPA, aan kweken waar differentiatie sterk gestimuleerd werd,

leidde tot sterke anti-differentiatie effecten. Dit werd aangetoond in verschillende *in vitro* en *in vivo* assays voor stamcellen en voorlopercellen. De combinatie van VPA en Li behield de onrijpe morfologie van de cellen en verbeterde de stamcel uitlezing na transplantatie. Bovendien zouden VPA en Li niet alleen differentiatie remmen van stamcellen, maar ook zelf-vernieuwing induceren van voorlopercellen. Verder hebben we aangetoond dat VPA en Li synergistisch de genetische code die specifiek is voor stamcellen en voorlopercellen kunnen behouden. Ook hebben we moleculen geïdentificeerd waarop VPA en Li hun anti-differentiatie effecten waarschijnlijk uitoefenen. Deze zogenoemde targets waren sterk gecorreleerd met elkaar tijdens normale stamceldifferentiatie, wat suggereert dat zelfvernieuwing en differentiatie van elkaar afhankelijke processen zijn. Deze beide processen dienen derhalve beïnvloed te worden in kweken om *ex vivo* stamcellen te expanderen. Dit maakt het mogelijk dat additieve/synergistische effecten van chemische verbindingen en andere mogelijke factoren die zelfvernieuwing beïnvloeden in een combinatorische screening ontdekt kunnen worden. Dit zou kunnen leiden tot nieuwe benaderingen om zelfvernieuwing en differentiatie van HSC te moduleren in *ex vivo* kweken voor stamcelexpansie. Bevindingen dat het lot van HSC kan worden gemoduleerd door de toevoeging van kleine chemische moleculen aan het kweekmedium is gunstig in het licht van *ex vivo* HSC expansie. Dit biedt, naast cytokines en ontwikkelingsfactoren aanvullende manieren voor controle over HSC zelfvernieuwing en differentiatie *in vitro*. Op dit moment, echter, is ons begrip van de moleculaire gebeurtenissen door chemische modulatie van HSC beperkt. Bovendien wordt VPA gebruikt in leukemie behandelingen als differentiatie therapie en derhalve is de specifieke identificatie van de effecten op stamcellen van belang om het potentieel van verbindingen zoals VPA begrijpen. In hoofdstuk 5 hebben we de genetische respons van de HSC op korte termijn VPA behandeling gekarakteriseerd, omdat de analyse van genen die door VPA worden beïnvloed (doelwitgenen) kan helpen om het volledige spectrum van de effecten ervan beter te begrijpen. Onze analyses toonden aan dat een korte, 24-uurs VPA behandeling verschillende cellulaire processen beïnvloed. Hieronder bevinden zich onder andere de activiteit van receptoren en hun signaal transductie, en veranderingen in HSC oppervlaktemarkers. Stamcellen kunnen worden geïdentificeerd door de aanwezigheid van de afwezigheid van specifieke markers op hun oppervlak. Bekende en veel gebruikte markers voor HSC, namelijk Sca-1 en Thy-1 werden geïdentificeerd al VPA doelwitgenen. Bijzonder is dat vooral Sca-1 in vele identificatiestrategieën voor muizen-HSC gebruikt wordt om het stamcelcompartiment van het meer gedifferentieerde myeloïde compartiment te scheiden. Stimulatie van hematopoïetische cellen met VPA zorgde voor behoud van Sca-1 expressie op de HSC's, maar ook werd Sca-1 opnieuw geïnduceerd op myeloïde progenitorcellen, die de expressie hadden verloren tijdens het proces van differentiatie. Hierdoor kregen de voorlopercellen weer het fenotype van een onrijpe stamcel. Stamcellen worden niet alleen gedefinieerd door de aanwezigheid van de specifieke markers maar vooral door hun functionele mogelijkheden. Belangrijk is dat de functionaliteit toegenomen was van stamcellen die de Sca-1 marker opnieuw verworven hadden en de mate van functionaliteit correleerde met de expressie van Sca-1. Maar re-inductie van functionele mogelijkheden was niet afhankelijk van het Sca-1 molecuul zelf, wat suggereert dat Sca-1 is een marker van cellen die stamcelachtige eigenschappen herwinnen. We kunnen dus zeggen dat VPA belangrijke effecten heeft op hematopoïetische

stamcellen en hun zelfvernieuwing en differentiatie kan induceren en mogelijk *ex vivo* kweken voor stamcelexpansie zou kunnen verbeteren. Hoewel HSC één van de best bestudeerde stamcel-systemen is, zijn op dit moment de geheimen van hun zelf-vernieuwing nog steeds niet bekend. En zo blijft ons vermogen om effectief HSC *in vitro* te manipuleren beperkt. Het bepalen hoe verschillende cel-intrinsieke en extrinsieke cel-signalen met elkaar geïntegreerd zijn en de identificatie van regulerende netwerken die HSC zelfvernieuwing controleren, lijkt cruciaal om succesvolle expansie protocollen te ontwerpen. Wat de toekomst van de *ex vivo* HSC expansie zal brengen is een intrigerende vraag. Wat zou de winnende combinatie van HSC expansie factoren zijn, die geschikt blijken voor klinische HSC protocollen? Op basis van de huidige kennis is het mogelijk om te zeggen dat het een combinatie van verschillende factoren zal zijn, zoals cytokines en kleine moleculen, waarvan precies de juiste verhouding nodig is voor de balans tussen proliferatie en zelfvernieuwing.

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SUMMARY IN ENGLISH

Hematopoiesis is the process of blood cell development and formation. Blood consists of many distinct and specialized cells that play an important role in an organism function, from oxygen supply (red blood cells) to all tissues in the body, protection and defence against pathogens (white blood cells) to blood coagulation (platelets) in case of injury. Many of these specialized, mature cells have a very short life span and they need to be continuously replaced. Approximately 10^{11}–10^{12} new blood cells are produced daily. This rapid cell turnover is dependent on a small population of blood-forming cells, namely hematopoietic stem cells (HSCs). HSCs can differentiate into any type of mature blood cell types, but they can also re-produce themselves in a process of self-renewal to maintain the stem cell pool. The balance between these two processes is crucial for an organism as it enables rapid and robust response to physiological stresses, including blood loss, infection and injury (multilineage differentiation) and it maintains lifelong blood production (self-renewal). The properties of HSCs render these cells an attractive source for a wide range of stem cell-based therapies where restoration of blood cells or introduction of normal hematopoiesis is needed. This can be the case in the treatment of cancer, bone marrow failure, immunodeficiencies and genetic disorders, where HSC transplantation can be a life-saving procedure. However, each human is different and in order for a transplantation to be successful the HSC from donor and patient have to be matched immunologically. This will prevent rejection of the transplanted cells and immune reactions from the graft against the recipient. The low availability of suitable, matched HSC from bone marrow and mobilized peripheral blood restricts their use. An alternative approach, allowing the use of more rapidly available mismatched donors, involves the use of umbilical cord blood (UCB). However, the stem cell number in a single UCB unit is not sufficient for transplantation into an adult, limiting the use of UCB. Therefore much research focuses on amplification of HSC numbers *ex vivo* (outside the organism), prior their transplantations. Nowadays we can treat only one patient with the HSCs from a single UCB unit and the challenge is to expand undifferentiated HSCs (via stimulation of self-renewal) in numbers sufficient for therapy of adult patients or even several patients. The success of *ex vivo* stem cell expansion protocols is dependent on our ability to manipulate the behavior of HSCs; to stimulate cell division with concomitant maintenance of an undifferentiated cell state. The development of such protocols requires the understanding of the mechanisms governing HSCs self-renewal and differentiation decisions, and ultimately defining key factors stimulating HSCs self-renewal divisions. In this thesis we aimed to explore the potential of distinct factors to regulate HSCs self-renewal and differentiation with the ultimate purpose of *ex vivo* stem cell expansion. During cell division the HSC has to choose between self-renewal and differentiation, and the balance between these two cell fates determines the outcome of *ex vivo* expansion culture. The fate of HSC is regulated by signals from both inside (intrinsic) and outside (extrinsic) of the cell. In chapter 3 we aimed to identify new intrinsic regulators of hematopoiesis. Cellular fate is programmed in the DNA, a genetic code of the cell that is translated to RNA levels and further to protein level. Genetic code of the cell can be influenced by other intrinsic molecules, such as non-coding RNAs, including microRNAs. MicroRNAs are short RNA molecules that can bind to RNA and inactivate the partner RNA so that RNA is not functional. In chapter 3 we investigated whether microRNAs

can influence the self-renewal of HSCs and thereby increase their numbers. To determine this we profiled microRNA expression patterns during hematopoietic differentiation in four distinct types of cells (stem cells, progenitor cells, and mature myeloid and erythroid cells) from two different strains of mice that differ in the frequency of HSCs. We focused on those microRNAs which first were active in stem cells and/or progenitor cells and less or hardly active in mature blood cells, and second that were most active in the mouse strain with the most blood-forming stem cells. These criteria could identify microRNAs that might be involved in stem cell fate regulation. This led to identification of an evolutionary conserved microRNA cluster consisting of miR-99b, let-7e and miR-125a. The function of this microRNA cluster was investigated by overexpressing the entire cluster and individual members of the cluster in the bone marrow cells. Subsequently, we tested the effect of overexpression in several stem and progenitor cell *in vitro* and *in vivo* assays (outside the organism or inside the organism, respectively). We found that cells overexpressing microRNA 99b/let7e/125a cluster were initially preserved in a stem cell-like state. Additionally, blood formation was disturbed in the mice transplanted with the miR-cluster cells. Detailed analysis of stem cell compartment from these mice revealed that HSCs overexpressing miR-cluster 99b/let-7e/125a were significantly compromised, resulting over time in stem cell exhaustion. The single microRNA, miR-125a was identified to be responsible for the majority of the miR-cluster 99b/let-7e/125a overexpression phenotypes. Also in this study a number of candidate partner RNA molecules of tested microRNAs were found. The microRNA 125a could bind to them and block their conversion to protein level, possibly influencing the stem cells fates. Since introduction of genetic material can result in undesired clinical outcomes following transplantation (e.g development of leukemia), stimulation of HSC amplification by extrinsic factors is the preferred tool for clinical *ex vivo* stem cell expansion. However, so far there has been limited success in preventing HSC commitment and differentiation with extrinsic factors and without genetic manipulation. One of the main problems of *ex vivo* expansion protocols is the inability to prevent stem cell differentiation with concomitant stimulation of cell proliferation. Recently it has been shown that small molecules could be used as new tools for manipulating cell fate decisions. Chapter 4 describes the potential of two small chemical molecules, lithium (Li, a simple cation) and valproic acid (VPA, a well-known histone deacetylase inhibitor) to promote maintenance of HSC in culture by inhibiting stem cell differentiation. The addition of the combination of these two compounds, Li and VPA, to differentiation-stimulating cultures of HSC resulted in strong anti-differentiation effects as shown by distinct stem and progenitor cell *in vitro* and *in vivo* assays. The combination of Li and VPA preserved immature morphology of the cells and improved the stem cell read-out following transplantation. Moreover, VPA and Li could not only inhibit differentiation of stem cells, but also induced self-renewal of progenitor cells. Further, we documented that VPA and Li synergistically preserved genetic code specific for stem and progenitor cell and we identified molecular targets of VPA and Li by which they could exert their anti-differentiation effects. Identified VPA and Li targets were strongly correlated with each other during normal hematopoietic differentiation, suggesting that self-renewal and differentiation seem to be strongly co-dependent. Thus, *ex vivo* stem cell culturing systems should target both processes to expand stem cells in culture. It is likely that combinatorial screening of

chemical compounds, and other potential self-renewal factors may uncover possible additive/synergistic effects to modulate HSC self-renewal and differentiation. This may ultimately lead to novel approaches in *ex vivo* stem cells culturing systems. Findings that HSCs fate can be modulated chemically by the addition of small molecules to the culture media is convenient in the light of *ex vivo* HSC expansion. This offers, next to cytokines and developmental factors, an additional level of control of HSC self-renewal and differentiation decisions *in vitro*. However, at present our understanding of the molecular events of chemical modulation of HSC is limited. Moreover, VPA has been used in leukemia treatment as a differentiation therapy and therefore detailed identification of its stem cell effects is of importance to understand the full potential of compounds such as VPA. In chapter 5 we characterized the genetic response of the HSC to short-term VPA treatment, since the analysis of VPA target genes may help to better understand the full spectrum of its effects. Our analyses revealed that short, 24h VPA treatment affected, among others, the activity of the receptor and the signal transducer activity and changes in the HSPCs surface marker profile. Stem cells can be identified by the presence or the absence of specific markers on their surface. Well-known and widely used HSPCs markers, namely Sca-1 and Thy-1 were identified as early VPA targets. Particularly, Sca-1 is included in many murine HSC identification strategies, separating the stem cell compartment from a more differentiated myeloid compartment. Stimulation of hematopoietic cells with VPA preserved Sca-1 expression on the HSCs, but it also re-induced Sca-1 marker on myeloid progenitor cells that had lost its expression in the process of differentiation, reverting those cells to a more immature stem cell phenotype. Stem cells are defined not only by the presence of the specific markers, but most importantly by their functional potential. Strikingly, the functionality of progenitor cells that re-acquired Sca-1 marker was also increased correlating with reversion of Sca-1. However, re-induction of cell functional potential was not dependent on Sca-1 marker, suggesting that Sca-1 is a marker of cells with stem cell-like phenotype. Collectively, we can say that VPA can induce important effects on hematopoietic stem and progenitor cell self-renewal and differentiation and could improve *ex vivo* stem cell expansion cultures. Although HSCs are the best studied stem cell system, at present the secret of their self-renewal remains hidden, and so does our ability to effectively manipulate HSCs *in vitro*. Defining how various cell-intrinsic and cell-extrinsic signals are integrated with each other and the identification of regulatory networks controlling HSCs self-renewal seems to be crucial to be able to design successful expansion protocols. What the future of *ex vivo* HSCs expansion will bring is an intriguing question. What would be the winning combination of HSCs expansion factors for *ex vivo* clinical HSC protocols? Based on present knowledge it is feasible to say that it would be a combination of several factors, such as cytokines and small molecules and just the right ratio to induce the perfect balance between proliferation and self-renewal.

SUMMARY IN POLISH
PODSUMOWANIE (DLA GENERALNEJ PUBLICZNOŚCI)

Proces wytwarzania i różnicowania się krwi zwany jest hematopoezą. Krew składa się z wielu różnych rodzajów komórek, które odgrywają ważną rolę dla prawidłowego funkcjonowania organizmu. Na przykład, czerwone krwinki (erytrocyty) dostarczają tlen to wszystkich pozostałych komórek w organizmie, białe krwinki (leukocyty) chronią przed i zwalczają zakażenia, a płytki krwi odpowiedzialne są za krzepnięcie krwi po zranieniu. Wiele z tych zróżnicowanych i wyspecjalizowanych komórek ma ograniczony czas życia i muszą być one ciągle odtwarzane. Codziennie we krwi produkowane jest około 10^{11}-10^{12} nowych komórek. Za produkcję nowych komórek, a także za ciągłość procesu tworzenia krwi odpowiedzialne są komórki macierzyste krwi. Komórki te posiadają bardzo szczególne właściwości, mianowicie mogę one zróżnicować się w każdy rodzaj komórek obecnych we krwi, ale mogą one również odtworzyć same siebie. Zdolność komórek macierzystych krwi do reprodukcji swoich identycznych kopii gwarantuje, że krew jest tworzona przez cały okres życia organizmu. Wspomniane dwie cechy charakterystyczne komórek macierzystych krwi, zdolność tworzenia wszystkich rodzajów komórek krwi i reprodukcji samych siebie, jest wykorzystywana w praktyce przy leczeniu chorób gdzie wymagane jest odtworzenie produkcji krwi, jak np, w leczeniu raka, niewydolności szpiku kostnego czy też w chorobach genetycznych. Pomimo ogromnego potencjału komórek macierzystych krwi, ich kliniczne zastosowanie jest ograniczone. Tradycyjnymi źródłami komórek macierzystych krwi są szpik kostny i krew. Jakkolwiek, aby leczenie (transplantacje) komórkami macierzystymi się powiodło, komórki pacjenta i dawcy muszą być identyczne lub bardzo podobne pod względem immunologicznym. Jednak każdy organizm, każdy pacjent jest inny i znalezienie odpowiedniego dawcy (bliźniaka genetycznego) jest bardzo trudne, a w wielu przypadkach niemożliwe. Alternatywnym źródłem komórek macierzystych krwi jest krew pępowinowa, która nie musi być dokładnie dopasowana genetycznie, aby leczenie się powiodło. Jednak i tutaj istnieją obecnie pewne ograniczenia. Ilość komórek macierzystych krwi w jednostce krwi pępowinowej jest niewystarczająca do terapii dorosłego pacjenta. Dodatkowo, ilość komórek macierzystych różni się znacznie pomiędzy dawcami. Namnożenie komórek macierzystych przed podaniem ich pacjentowi może znacznie poprawić użyteczność krwi pępowinowej, a także może umożliwić leczenie dorosłych pacjentów lub nawet kilku pacjentów komórkami pochodzącymi z jednej jednostki krwi pępowinowej. Obecnie wiele badań jest prowadzonych mających na celu namnożenie komórek macierzystych krwi *in vitro*, czyli poza organizmem, w naczyniu laboratoryjnym. Moja praca doktorska zajmuje się właśnie tym problemem. W rozdziałach tej książki opisane są badania skupiające się na lepszym scharakteryzowaniu i poznaniu w jaki sposób komórki macierzyste odtwarzają same siebie. Dodatkowo, nowe metody hodowli komórek macierzystych zostały zaproponowane. W celu zwiększenia liczby komórek, komórka musi się podzielić na dwie komórki, te dwie nowe komórki na cztery, itd. Sukcesem do namnożenia komórek macierzystych *in vitro* jest zdolność stymulacji podziałów gdzie początkowa komórka macierzysta będzie odtwarzała samą siebie wiele razy. Jeśli komórka się zróżnicuje, traci ona zdolność rekonstrukcji krwi podczas transplantacji. Jak dotąd nie zidentyfikowano optymalnych warunków stymulujących samoodnowę komórek macierzystych. Decyzje o losie komórki podczas jej podziału (samoodnowa lub różnicowanie)

są regulowane przez informacje genetyczną komórki i czynniki wewnątrz komórki, ale także czynniki z otoczenia komórki mogą mieć wpływ na jej los. W rozdziale 3 tej książki opisane są nowo odkryte „wewnętrzne" czynniki regulujące los komórki, tak zwane microRNA. W rozdziale tym zidentyfikowane zostały microRNA charakterystyczne dla komórek macierzystych krwi. Efekt wybranych microRNA (microRNA-99b/let7e/125a) na decyzje samoodnowy lub różnicowania komórek macierzystych krwi został tutaj zbadany. Zidentyfikowane microRNA początkowo zwiększyły wydajność transplantacji, jednak szczegółowe badania pokazały, że balans pomiędzy samoodnową a różnicowaniem był zaburzony, na korzyść różnicowania. Dodatkowo, mechanizm działa microRNA-99b/let7e/125a został zaproponowany. W rozdziale 4 zastosowana zostało inna strategia. Poprzez zewnętrzną stymulacje komórek próbowano zahamować różnicowanie komórek macierzystych w hodowli. Niedawno odkryta zdolność regulowania losu komórek macierzystych poprzez małe cząsteczki została tutaj wykorzystana. W rozdziale 4 pokazano, że lit i kwas waproinowy, w połączeniu, mogą zahamować lub też opóźnić różnicowanie komórek macierzystych krwi w hodowli. Anty-różnicujące efekty litu i kwasu waproinowego zostały pokazane *in vitro* jak i *in vivo*. Synegistyczny mechanizm działa litu i kwasu waproinowego został zidentyfikowany w szeregu testów. Genetyczne podłoże efektu hamującego różnicowanie zostało także tutaj zaproponowane. W rozdziale 5 mechanizm działania kwasu waproinowego na komórki macierzyste krwi został zbadany i opisany bardziej szczegółowo. Do tej pory do hodowli komórek macierzystych używano klasycznych czynników wzrostowych dla tych komórek. Opisana tutaj praca pokazuje, że małe cząsteczki także mogą regulować zachowanie komórek macierzystych krwi w hodowli, i sugeruje, że testowanie kombinacji rożnych czynników może doprowadzić do rozwoju nowych, lepszych systemów do hodowli komórek macierzystych krwi.

Wiele lat badań wciąż przed nami, aby poznać tajemnice mechanizmu samoodnowy komórek macierzystych i odkrycie optymalnych warunków do ich hodowli. Na podstawie tego co wiemy do tej pory, można przypuszczać, że będzie to kombinacja wielu różnych czynników i cząsteczek, a także odpowiedniego balansu pomiędzy nimi. Interesującym i ekscytującym pytaniem jest jak przyszłe systemy hodowli komórek macierzystych będą wyglądały i jakie czynniki hodowli będą zwycięzcami wyścigu o nielimitowane namnożenie nieróżnicowanych komórek macierzystych krwi.

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